1941

The effect of controlled pH upon the production of chemicals in several fermentations

Richard James Hickey
Iowa State College

THE EFFECT OF CONTROLLED pH UPON THE PRODUCTION OF CHEMICALS IN SEVERAL FERMENTATIONS

by

Richard James Hickey

A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject Biophysical Chemistry

Approved:

Signature was redacted for privacy.

In charge of Major work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State College
1941

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. ACKNOWLEDGEMENTS

The author wishes to take this opportunity to express his sincerest gratitude to Dr. Ellis I. Fulmer for suggesting this topic, and to him and to Dr. Leland A. Underkofler for their most valuable and generous assistance and advice during the course of this investigation. Mr. G. Warren Buckaloo's assistance in the development of the optimum media was most valuable, and Mr. Thomas M. Lees' original ideas and assistance especially along mechanical lines and in analytical work were sincerely appreciated.

II. INTRODUCTION

Glycerol has not been produced on a commercial basis by fermentation in normal times with any great degree of economic success. It was successfully produced by fermentation in Germany, however, during the World War of 1914-1918 for use in the manufacture of explosives. Such a production was necessary for a national emergency where the end justified the means in spite of the high cost of manufacture. The Connstein and Lüdecke (1921)(1924) fermentation process in Germany produced glycerol from beet sugar in the presence of sodium sulfite. About twenty-four factories produced approximately one thousand tons of glycerol per month by this rather inefficient process. Smaller amounts of glycerol were produced in the United States following the success of the Germans, but sodium carbonate was used in place of sodium sulfite. This alkaline fermentation method was patented by Neff (1918). In England, the Cooking and Lilly (1922) process was used to some extent. It is similar to the method of Connstein and Lüdecke except that sodium bisulfite is employed along with the sodium sulfite in order to increase the yield of glycerol. It is claimed to be a means of producing practically a theoretical yield of glycerol by fermentation.

In most of the glycerol fermentation processes sodium salts are added to increase the glycerol yield. The relatively high solubility

of these sodium salts causes certain difficulties in the recovery of the glycerol from the mash. The glycerol is not easily distilled in the presence of such salts without some decomposition or etherization. More efficient recovery methods have been developed, however, in recent years. Glycerol extraction methods are usually rather involved, expensive, or impractical.

In order for fermentation glycerol to compete on an economic basis with that produced as a by-product of the soap industry, the method of formation of the glycerol must be very efficient and also the procedure of recovery must not require excessive expense to operate. The percentage recovery of the available glycerol should be at least 90 percent. The utility and value of the by-products must also be considered, as these might well be the factors which determine the success or failure of the process. Recent methods for the synthesis of glycerol from petroleum products appear to have promise of success. On the other hand, there are very large quantities of blackstrap molasses available as a source of sugar for fermentation at very low cost. Corn or corn sugar might also be potential carbohydrate sources.

Glycerol is a very useful commodity in an exceptionally wide variety of fields. It is produced almost exclusively by the soap industry at present. Since this production is limited by the quantity of the soap produced, glycerol is not used as widely in industry as it might be if more were available at a more reasonable cost to the

consumer. If a consumer were to use very large quantities of glycerol, he would no doubt desire practically an unlimited quantity to be available at a stable price level. He would not consider expansion of an industry or process which would be controlled by the limited availability of one component. He could get no more than a fixed amount of glycerol from the soap industry. If he wanted more than the soap industry could supply, he would, no doubt, have to turn to either the fermentation processes or to the synthetic processes. The price of his glycerol would then probably increase to such an extent that his process would no longer be industrially possible without economic difficulties. Synthetic glycerol is still in experimental stages, but according to Williams and associates (1940)(1941) it can be made at approximately the market price. Fermentation glycerol is as yet too expensive for widespread manufacturing uses. Use as an antifreeze in automobiles would require an inexpensive product. The plastic industry could employ quantities of glycerol if it were available at a low enough price. A great number of other possible uses are at hand. The petroleum industry is developing methods for the production of long chain fatty acids from petroleum hydrocarbons and it is desired that these might be converted into glyceryl esters or fats to be used in the food industry.

There has been a large amount of research done on the subject of the production of glycerol by fermentation and it might appear to some that the glycerol fermentation has very little prospect of

becoming a thriving industry. That is a matter of conjecture which the future will prove. If the results of this work contribute something of value to either the academic or industrial fields, then some of the aims will have been realized. It should be borne in mind, however, that although a large part of the experimental work has been done on the glycerol fermentation, many of the principles, methods, and theories might well be applied to other fermentations with quite satisfactory results. Some of the work is concerned with the relationship of pH to the production of glycerol by the alteration of a yeast fermentation from its normal course wherein the pH is usually about 3. The pH is altered toward about 9, or the extreme limit of endurance of most strains of yeast. Glycerol production is thereby greatly increased. There would be interest in determining the glycerol yield as a mathematical function of the pH of the medium. Then ionic effects on the course of the reaction could possibly be studied to more advantage.

The purposes of this thesis are to study certain fermentations under abnormal pH conditions, to obtain relations between pH and the yields of the products of dissimilation of certain carbohydrates in the presence and in the absence of certain fixing agents, and to obtain methods for glycerol production by fermentation such that the added salts or other compounds may be easily removed by simple methods such as filtration or distillation or by combinations of the two in order to assist in making the glycerol fermentation an

industrial reality. This thesis is concerned primarily with studies on the glycerol fermentations. Special attention is given to the relation between pH and the yields of various dissimilation products of carbohydrates in the presence or absence of certain aldehyde fixing agents. Attention is also paid to the development of glycerol fermentation methods which should allow greatly simplified methods of glycerol recovery following the fermentation.

III. REVIEW OF PREVIOUS INVESTIGATIONS

A. Historical

Glycerol, also called glycerine, or propan-1,2,3-triol, is a thick liquid which is practically colorless and odorless. It is rather sweet tasting and may be used as a food. Literature on the subject of glycerol in practically all its aspects has been very ably covered up to 1928 in the monograph by Lawrie (1928). Because of the availability of this monograph, topics and references up to 1928 will be mentioned very briefly or omitted altogether unless the subject material is very pertinent to the investigations made for this thesis. The brief history in the following paragraph was taken directly from Lawrie (1928).

In 1779 Scheele prepared glycerol by heating a mixture of olive oil and litharge. Later, in 1784, he showed that this material could be obtained from a variety of oils and fats both of animal and of vegetable origin. The procedure of Scheele was about the only method of commercial production of glycerol for many years. In 1823 Chevreul published work which led to the alkaline hydrolysis of fats and oils as a method of glycerol production. Later it was found by Tilghman, in 1853, that if fats were mixed with water and heated under pressure without steam formation, saponification occurred although alkalis were

not present. In 1856 Wilson improved the procedure by means of a steam distillation method. Other improvements in the production of glycerol from fats and oils followed later. Glyceryl trinitrate, or "nitroglycerine", was discovered in 1846 by Sobrero. The work was published in 1847. Nobel demonstrated its explosive value in 1863, and by absorbing it in Kieselguhr he developed dynamite in 1868. In 1875 he developed blasting gelatin.

Glycerol has been prepared by a variety of synthetic organic methods. One of the earlier methods was that of Wurtz (1857) who treated 1,2,3-tribromopropane with silver acetate and obtained triacetin which gave glycerol on alkaline hydrolysis. More recently, methods have been proposed for the production of glycerol from natural or cracked petroleum products such as propane and propylene. Although many of the older synthetic methods had mainly historical and academic value and little industrial possibilities, some of the newer methods are considered as possible glycerol sources. Williams and associates (1938)(1940)(1941) have described a method involving a high pressure chlorination of propylene to form allyl chloride. The allyl chloride is converted to allyl alcohol by a sodium hydroxide treatment. Hypochlorous acid is added to the unsaturated group to form the monochlorohydrin which is then hydrolyzed to glycerol. The above method has been operated recently on a small scale at a profit. Methods of this type have shown great promise. Thus with both the glycerol and fatty acids available from petroleum

products, fats and oils similar to the natural ones have been prepared from petroleum products.

The fact that glycerol is a product of fermentation was first noted by Pasteur (1858). He was studying pure yeast cultures in connection with the production of wines and beers, and on quite complete analyses of the fermented mashes he found that about 3.5 grams of glycerol were normally formed from every 100 grams of sugar. For quite a while this fact, though published, made very little impression on scientists in general. It was not until about 1911 that Neuberg and his co-workers began to publish some of the results of their investigations on the problem of the alcoholic fermentation mechanism (1913)(1915)(1918)(1919). Their conclusions as to the mechanism of the dissimilation are shown in the following equations:

(1) $C_6H_{12}O_6 - 2H_2O \longrightarrow C_6H_8O_4$ (methyl glyoxal aldol)

(2) $C_6H_8O_4 \longrightarrow 2CH_2{=}C(OH)CHO$ or $2CH_3COCHO$ (methyl glyoxal)

(3) $CH_2{=}\underset{OH}{C}{-}CHO + H_2O + H_2 \longrightarrow C_3H_5(OH)_3$ (glycerol)

$CH_2{=}\underset{OH}{C}{-}CHO + O \longrightarrow CH_2{=}\underset{OH}{C}{-}COOH$ (pyruvic acid enol)

(4) $CH_2{=}\underset{OH}{C}{-}COOH \longrightarrow CO_2 + CH_3CHO$ (acetaldehyde)

(5) $CH_3COCHO + O \longrightarrow CH_3COCOOH$ (pyruvic acid)

$CH_3CHO + H_2 \longrightarrow CH_3CH_2OH$ (ethyl alcohol)

Many mechanisms for sugar dissimilations have been advanced, but at present the most generally accepted mechanism is that of Embden, Meyerhof, and Parnas. A review and discussion by Werkman (1939) excellently covered this subject and certain related topics. A comprehensive bibliography was included with this work.

Neuberg's studies led him to the idea of fixing the acetaldehyde formed in the fermentation by agents such as a sulfite or an organic agent, dimedon. Later, Kobel and Tychowski (1928) fixed the aldehyde by means of carbaminic hydrazide or thiocarbaminic hydrazide during fermentation. Neuberg used sodium sulfite for the most of his experiments, but he also employed other sulfites such as those of calcium, magnesium, and zinc. Since the bisulfite radical is concerned with the fixation of acetaldehyde, Neuberg suggested the following theoretical reaction:

$$C_6H_{12}O_6 + Na_2SO_3 + H_2O \longrightarrow CH_3CHO.NaHSO_3 + NaHCO_3 + C_3H_8O_3$$

Neuberg (1920) considered that the sugar dissimilation by yeast takes place in three forms, namely:

(1) $C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$ (regular alcoholic form)

(2) $C_6H_{12}O_6 \longrightarrow CH_3CHO + CO_2 + C_3H_8O_3$ (sulfite mechanism)

(3) $2C_6H_{12}O_6 + H_2O \longrightarrow 2CO_2 + CH_3COOH + C_2H_5OH + 2C_3H_8O_3$ (alkaline mechanism)

Reaction (1) above is generally known as the Gay-Lussac reaction. Form (3) was considered as a continuation of form (2) wherein the alkali caused the acetaldehyde to undergo the Cannizzaro reaction to form the ethanol and acetic acid. Discussion and data which substantiated Neuberg's claims are adequately expounded in Lawrie's monograph (1928).

According to Anderson (1938), Meyerhof considered the following reactions to occur in the dissimilation of a hexose by yeast:

(A) Hexose diphosphate + $C_6H_{12}O_6 + 2Na_2HPO_4$ → $\begin{matrix}CHO\\|\\CHOH\\|\\CH_2OPO_3Na_2\end{matrix}$ (glyceraldehyde phosphate) → $\begin{matrix}CH_2OH\\|\\CHOH\\|\\CH_2OPO_3Na_2\end{matrix}$ (α-glycero-phosphate) + $\begin{matrix}COOH\\|\\2CHOH\\|\\CH_2OPO_3Na_2\end{matrix}$ (phospho-glycerate)

(B) $\begin{matrix}COOH\\|\\2CHOH\\|\\CH_2OPO_3Na_2\end{matrix}$ → $2Na_2HPO_4$ + $\begin{matrix}COOH\\|\\2\,CO\\|\\CH_3\end{matrix}$ (pyruvic acid) → 2_4CO_2 + $\begin{matrix}CHO\\|\\2\,CH_3\end{matrix}$

(C) $C_6H_{12}O_6$ + $2Na_2HPO_4$ → $\begin{matrix}CHO\\|\\2CHOH\\|\\CH_2OPO_3Na_2\end{matrix}$ $\xrightarrow{2\,CH_3CHO}$ $\begin{matrix}CH_2OH\\|\\2\,CH_3\end{matrix}$ + $\begin{matrix}COOH\\|\\2CHOH\\|\\CH_2OPO_3Na_2\end{matrix}$

Meyerhof considered parts (A) and (B) to be the "Initial phase", and part (C) was considered as the "Stationary Condition" which was supposed to be the situation after the fermentation had started through the "Initial phase", and enough acetaldehyde had formed to

act as the hydrogen acceptor in place of the glyceraldehyde phosphate which is the hydrogen acceptor in the absence of acetaldehyde.

For extensive discussions on fermentative and respiratory mechanisms, and on the roles of various agents in metabolism, one should refer to the books by Stephenson (1939), Oppenheimer and Stern (1939), Anderson (1938), and Prescott and Dunn (1940).

Connstein and Lüdecke (1919), after considering the earlier work of Neuberg, studied the glycerol fermentation with the view of producing glycerol on a commercial basis. They decided, on a theoretical basis, that alkaline fermentations should result in increased glycerol production. This was found to be a fact, and their observations were instrumental in the derivation of Neuberg's (1919)(1920) third fermentation mechanism. Some of their observations are noted in Table 1. By percent of glycerol produced is meant

TABLE 1

GLYCEROL YIELDS USING ALKALINE SALTS IN THE FERMENTING MEDIUM

Alkaline Salt	Parts by Weight		Glycerol Produced (% on sugar)
	Salt	Sugar	
No salts (control)	00	100	3.0
Sodium acetate	30	100	9.5
Sodium phosphate (sec.)	46	100	11.0
Sodium phosphate (sec.)	70	100	15.6
Sodium bicarbonate	14	100	12.7
Ammonium carbonate	10	100	13.4

(Adapted from Lawrie (1928))

the percent of the weight of the sugar found as glycerol; that is, a 10 gram yield of glycerol from 100 grams of sugar is considered as a 10 percent yield. The theoretical yield of glycerol from either the alkaline or the sulfite mechanism is very close to 51 grams from 100 grams of hexose.

Unfortunately, the pH values of these media were not given. The absence of pH data has been typical of a large number of publications. Since certain ions, such as the phosphate ion, are intimately concerned with the sugar breakdowns, it is rather uncertain to what extent the effects are due to the abnormal ion concentrations, and to what extent the effects are pH functions.

It was found that secondary infections caused certain difficulties in the alkaline fermentations; however, in sulfite fermentations the use of high concentrations of sodium sulfite stopped most

TABLE 2

THE EFFECT OF INCREASING AMOUNTS OF SODIUM SULFITE IN YEAST FERMENTATIONS ON GLYCEROL YIELDS

Parts by Weight		Glycerol Produced
Sodium Sulfite	Sugar	(% on sugar)
40	100	23.1
67	100	24.8
80	100	27.3
100	100	30.1
120	100	33.0
150	100	34.6
200	100	36.7

(Adapted from Lawrie (1928))

of the infections. Table 2 shows the effect of increasing amounts of sodium sulfite on the yield of glycerol.

For the alkaline fermentation, Neuberg also used other reagents than those mentioned above. Some of his reagents are noted below:

TABLE 3

REAGENTS CAUSING NEUBERG'S FIRST AND THIRD MECHANISMS TO OCCUR

First Mechanism	Third Mechanism
Aluminum Hydroxide	Sodium Carbonate
Ferric Hydroxide	Sodium Bicarbonate
	Potassium Carbonate
	Potassium Bicarbonate
	Magnesium Oxide
	Sodium Phosphate (normal)
	Sodium Phosphate (secondary)
	Zinc Hydroxide

(Adapted from Lawrie (1928))

Individual processes and patents are taken up in the sections which follow.

B. Glycerol Formation by the Sulfite Fermentation Methods

Preliminary work on the sulfite process of fermentation was mentioned in previous sections. The actual processes, many of which were patented, are now presented in greater detail.

1. The Connstein and Lüdecke process

As was previously mentioned, the Connstein and Lüdecke (1919) (1921)(1924) process was carried on in Germany during World War I in about twenty-four factories, and about one thousand tons of glycerol were produced each month in this manner. After the war the process was patented in many countries and the patent references are noted by Lawrie (1928). The following five claims are made in their United States patent (1924):

1. The process for manufacturing propantriol which consists in adding alkaline sulfites (until alkaline reaction) and yeast to sugar and then allowing the mixture to be fermented.

2. The process for manufacturing propantriol which consists in adding alkaline sulfites (until alkaline reaction) and yeast to sugar, causing the sugar to be fermented in the presence of alkaline sulfites, separating the yeast and adding the separated yeast and alkaline reacting substances to sugar, whereupon the process is repeated.

3. The process for manufacturing propantriol which consists in adding alkaline sulfites (until alkaline reaction), neutral salts of magnesium in a higher amount than necessary as yeast nutrients, and yeast to sugar, and causing the sugar to be fermented.

4. The process for manufacturing propantriol which consists in adding alkaline sulfites (until alkaline reaction) and yeast to sugar, causing a portion of the sugar to be fermented, adding new portions of sugar and causing the sugar to be fermented.

5. The process of producing glycerol, which consists in fermenting a solution of fermentable sugar in an alkaline reacting medium.

It was claimed that neither the kind of sugar nor the yeast variety had any appreciable effect on the outcome of the fermentation. This claim was disputed by other investigators, such as Gehle (1922) and McDermott (1925), especially with regard to the strain or variety of yeast. Saccharomyces ellipsoideus was employed by some investigators in preference to Saccharomyces cerevisiae strains, although the S. ellipsoideus fermentations were somewhat slower.

Connstein and Lüdecke (1921)(1924) claimed success using both crude and refined sugars, and also molasses. They employed the yeast from one fermentation to seed the next for eight fermentations with apparent success. Objections to this method appeared subsequently. Connstein and Lüdecke presented the following data on the use of the yeast of one fermentation as the inoculum for a following fermentation:

TABLE 4

EFFECT ON GLYCEROL YIELD OF USING YEAST FROM ONE FERMENTATION AS SEED FOR THE NEXT

Times Yeast is Used	Glycerol Yield (% on sugar)
1	18.8
2	21.4
3	22.9
4	22.8
5	22.3
6	20.9
7	19.9
8	21.2

(From Lawrie (1928))

Details of the process are, for example: To ten liters of water were added one kilogram of sugar and 100 grams of fresh yeast, along with nutrient salts of potassium, phosphorous, magnesium, and nitrogen. Finally 400 grams of sodium sulfite (anhydrous) were also added. The medium was shaken quite well and was incubated at 30° centigrade for 48 to 60 hours. It was noted that there was not an appreciable increase in the alkalinity of the mash since the carbon dioxide and acetaldehyde reacted, supposedly, with the sodium sulfite to form some sodium bicarbonate and also some bisulfite-aldehyde addition product. The volatiles were removed by distillation, after which calcium chloride and lime were added to the remaining medium to precipitate the sulfite. The filtrate so obtained was treated with sodium carbonate to precipitate the excess of soluble calcium salts. The resulting filtrate was concentrated by

evaporation, and the syrup thus obtained was glycerol containing sodium chloride and some other soluble materials. The purified glycerol was procured by steam distillation. It was regularly noted that as the amount of sulfite was increased, the yield of glycerol and acetaldehyde increased, and that of the alcohol decreased. Neuberg's first and second schemes both occur in this process. Experimental data substantiated Neuberg's ideas quite well.

Fermentations using other salts were studied, but the glycerol yields were lower than those obtained by the sulfite fermentations. Results are shown in Table 5.

TABLE 5

YIELD OF GLYCEROL BY FERMENTATION WITH VARIOUS SALTS

Salt	Weight (% of sugar wt.)	Glycerol Yield (% on sugar)
$CaCl_2$	40	8.5
NH_4Cl	30	7.5
NaCl	19	8.0
Na_2SO_4	24	6.7
Na_2SO_4	48	8.0
$NaNO_3$	34	5.5
$FeSO_4$	60	11.8
$FeSO_4$	90	12.5
$FeSO_4$	120	13.1
$Al_2(SO_4)_3$	39	9.4
$Al_2(SO_4)_3$	44	11.6
$Al_2(SO_4)_3$	80	13.3

(From Lawrie (1928) and Connstein and Lüdecke (1921))

The above data showed that the acid reacting salts (e.g. ferrous sulfate and aluminum sulfate) in increasing concentrations increased the yield of glycerol in contrast to the idea that increased alkalinity alone increased the glycerol yields in the absence of sulfites.

2. The Cocking and Lilly process

Cocking and Lilly (1922) developed a process which was claimed to produce glycerol according to Neuberg's second scheme in practically theoretical quantities. The purpose of this work was to improve the method of Connstein and Lüdecke in order to increase the glycerol yields. It was considered desirable to introduce a certain concentration of bisulfite ions in the medium containing sodium sulfite at the onset of the fermentation. This was done in order to fix the acetaldehyde as it formed at the start of the fermentation and not to wait until acids formed by fermentation reacted with the sodium sulfite to form some bisulfite. Unfortunately, large amounts of bisulfite ions are undesirable during a successful glycerol fermentation by certain yeasts because of the antiseptic action. Cocking and Lilly found that sodium sulfite and sodium bisulfite could be used in combinations in which the resulting solution was nearly neutral to litmus. There was then no serious antiseptic action. Thus the aldehyde was fixed at a much earlier stage in the fermentation than occurred in the sulfite fermentation

of Connstein and Lüdecke (1921)(1924). It was claimed that the time of fermentation using the mixture of acid and normal sulfites was about half the time of fermentation when the normal sulfite alone was used. The time of the fermentation was usually about six days, and the temperature was held at about 35° to 37° centigrade. The glycerol yields usually approximated 45 percent of the sugar weight. It appeared that the higher yields of glycerol were associated with longer times of fermentation.

3. Other methods

The work of Neuberg on sugar fermentations by yeasts in the presence of sulfites was confirmed by Gehle (1922). He noted that with increasing sulfite concentrations there was an increasing change in the products of fermentation, and that the alteration practically ceased when there were about 60 parts of sodium sulfite to 100 parts of sugar. He also observed that different types of yeast did not appreciably affect the qualitative and quantitative dissimilation, although there was a rather noticeable difference in the degree of resistance of the various yeasts to the sulfite.

Barbet (1928) made use of sulfur dioxide for the sulfite fermentation. To a molasses mash was added some sulfur dioxide previous to inoculation to the extent of about two grams per liter. The mash was then seeded and fermentation proceeded with the addition of sulfited medium continuously or intermittently in quantities not

great enough to exhibit excessive toxic effects. The fermentation was allowed to complete itself, and a considerable amount of glycerol was found. It is important to note that, in effect, an acid, sulfurous acid, was used as an agent to increase the glycerol yield in a yeast fermentation and not an alkaline reacting material. It has been generally considered that in most any acid medium a more or less typical alcoholic fermentation must occur. Thus Barbet obtained increased glycerol yields by the use of an acid added to a fermenting mash. Actual yields were not given by Barbet in his patent.

There are numerous other patents concerning the use of salts, neutral and otherwise, along with sulfites to produce glycerol. According to Lawrie (1928) some patents claimed that in addition to sodium sulfite, about 0.2 to 1 percent of the weight of sugar should be added in the form of salts of strongly reducing sulfur acids such as "hydrosulfates" and "sulfoxylates." This addition was claimed to give glycerol yields greater than those obtained by the use of sodium sulfite alone. Ferrous and manganese sulfates were considered "catalysts" to increase glycerol formation. The addition of more sugar to the mash at its point of greatest fermentation activity with or without salt additions was also described. Further details are found in the monograph of Lawrie (1928).

In Russia, Golovin (1927) described a fermentation using a bisulfite. The fermentation was complete in 48 hours, and on the

sugar weight basis yields of 20 percent glycerol, 20 percent alcohol, and 8 percent acetaldehyde were claimed.

Lüdecke and Lüdecke (1929) obtained a patent on a sulfite method of refermentation following a distillation of the first fermentation medium. The temperature was held at 30° to 35° centigrade for two days. Magnesium and nickel sulfates were added. Eight kilograms of molasses gave two kilograms of raw glycerol or 960 grams of pure glycerol. About 24 to 27 percent of glycerol was obtained from pure sugar. Tomoda (1928) observed that in the presence of sodium sulfite, the alcoholic and glycerol fermentations occurred parallel from start to finish. Mathematical relations were given.

Penkovski (1929) studied the sulfite fermentation of sugar beets and obtained 20 percent glycerol, 17.5 percent ethanol, and 8 percent acetaldehyde. In this year, Tomoda (1929) studied the velocity of the fermentation in the presence of sulfite. He also studied the formation of 2,3-butylene glycol and acetic acid during a sulfite glycerol fermentation. Imperial Chemical Industries, Ltd. and Lilly (1930) improved on the sulfite fermentation of carbohydrates and molasses.

Giordani (1932) made a theoretical study of the sulfite fermentation of Constein and Lüdecke. He agreed that bisulfites increased glycerol yields by combining with the acetaldehyde first formed in such a way as to prevent its further conversion to acid and alcohol. A maximum yield of 25 percent glycerol was found when fermentation was carried out in the presence of 20 percent sodium bisulfite.

Takahashi and Asai (1933) studied both the sulfite and alkaline dissimilations of sugar by means of 23 varieties of Mucor. Normally, glycerol was produced to the extent of 3.8 to 9.0 percent for the assimilated sugar; production was approximately parallel to that of alcohol. It was found that the addition of sodium bisulfite or sodium carbonate greatly increased the glycerol yield. The optimum concentrations of these salts were found to be, respectively, 6 and 4 percent. The corresponding yields of glycerol were 21.5 and 23.5 percent respectively, for the assimilation of glucose.

Lilly (1935) described a method for a fermentation wherein soluble sulfites and bisulfites such as the sodium salts were added to a fermentation medium in a manner similar to the original Cocking and Lilly (1922) process, keeping the medium about neutral. However, there were added to the fermenting mash at intervals small quantities of regenerated sulfite-bisulfite solution containing the glycerol of a previous dissimilation.

The Russian investigators, Kurbatova and Shakin (1936), reported that they used yeast of a sulfite fermentation repeatedly without loss of fermenting activity, provided that a sulfite-free growth of a culture is interposed between each sulfite fermentation. It was recommended that the yeast cells be separated from the sulfite medium as soon as fermentation is complete.

Iwata (1936) reported on a process for obtaining glycerol from cane juice by fermentation. He added salts and a quantity of sodium

sulfite to the cane juice and inoculated with the molasses yeast, Saccharomyces formosensis nov.sp. The best yield was 27.47 percent glycerol calculated on the fermentable sugars. With a mixture of sodium sulfite and sodium bisulfite he obtained a yield of 17.91 percent of the sugar weight as glycerol. The pH was kept from 7 to 8.5 by means of sodium carbonate or sodium sulfite. For recovery of the glycerol, Iwata neutralized with sulfuric acid, distilled volatile materials, evaporated to dryness, and extracted with four volumes of absolute alcohol to one volume of carbon tetrachloride. The glycerol left after the distillation of solvents was 70 to 80 percent pure.

Lüdecke (1938) obtained a patent on a method whereby sugars or crude sugar solutions obtained by condensation of formaldehyde preferably with the assistance of natural or artificial light were fermented to form glycerol.

Rao (1937) studied the waste cane molasses fermentation to form glycerol. He fermented the molasses by means of S. cerevisiae in the presence of alkaline carbonates, bicarbonates, and especially sulfites. He obtained a 10 to 15 percent yield of glycerol calculated on the sugar.

The Norddeutsche Hefeindustrie A.-G. (1938) obtained a patent on the glycerol fermentation in which about 3 percent sodium chloride was considered important in addition to the alkali sulfite. The example described a medium containing sugar, sodium chloride, sodium

bicarbonate, ammonium sulfate, magnesium sulfate and yeast. The temperature was maintained at 37° centigrade, and the pH of the medium was 7.2 to 7.5. Haehn (1938)(1940) developed a new method for glycerol production. His contention was that aeration in the presence of oxidation catalysts, e. g., iron or manganese salts, was advantageous for glycerol production. Sodium bisulfite was used, along with ammonium phosphate and magnesium sulfate in the fermentations. Air was supplied through a porous cup, and the pH was kept "within limits". After eight hours, the glycerol was recovered and found to be 25 to 30 percent of the sugar weight. A top yeast was used which was separated and reused. In other examples "mold yeasts" were employed such as the genera Torula, Mycoderma, Pichia, and Willia. An experiment using a Mycoderma strain yielded 30 to 34 percent glycerol after 23 hours at 34° centigrade.

Hesse (1935) indicated that maximum yields of glycerol and acetaldehyde were obtained when fermentation occurred in the presence of the maximum amount of sodium sulfite that the yeast could tolerate. When 33 grams of sodium sulfite were used per 100 grams of sugar, there were obtained 11.90 percent of acetaldehyde and 22.37 percent of glycerol. When 100 grams of sulfite were used, the yields were 18.96 percent aldehyde and 36.99 percent glycerol.

A short review on the subject of blackstrap molasses as a raw material for the production of glycerol by fermentation was made by

Owen (1937)(1939). Statistical and economic references were made. He considered the presence of bio-colloids and the like to be of quite unsuspected importance in a dissimilation such as the glycerol fermentation. Owen stated (1937)(1939):

> It has been found that the addition of minute amounts of the finely divided and highly dispersed absorbent materials like clay, bone-black, activated carbon, etc., not only accelerate the rate of fermentation of sugars by yeast, but also provide conditions for the efficient conversion of sugars into alcohol in solutions whose densities or sugar concentrations would render it impossible in the absence of these bio-colloids.
>
> While many explanations have been advanced for this benign action of these substances, all of the investigators have concurred in the opinion that much of it is to be attributed to the activation of the hydrogen liberated in the reaction, and its consequently more effective action in reducing the acetaldehyde to alcohol. In so far as this is true, it represents an exactly opposite function of these substances to that of alkali or sulfites in immobilizing the acetaldehyde, and thereby preventing its reduction to alcohol, and resulting in the formation of glycerin from the sugar molecule.

C. Glycerol by the Alkaline Fermentation Methods

The production of glycerol by means of yeast fermentations of sugars in an alkaline medium has not been considered as a commercial possibility as long as has the sulfite method. The fact that the products associated with the glycerol made by the alkaline methods are ethanol and acetic acid, as compared to acetaldehyde by the sulfite method, might have great influence on the value of the fermentation. Ordinarily, ethanol and acetic acid are more desirable than is acetaldehyde. An alkaline process for the production of fermentation glycerol was developed in the United States about the time of the World War I.

1. The Eoff process.

During the World War I the price of glycerol rose quite rapidly to about sixty cents per pound. The United States Treasury Department was notified by Dr. Alonzo E. Taylor that glycerol was being produced in Germany by a fermentation method. Soon three government laboratories started research on this problem. Eoff, Linder, and Beyer (1919) discovered that the addition of certain alkaline reagents to a fermentation medium caused a rather large increase in the amount of glycerol formed from a given amount of sugar. Reagents used were sodium and potassium carbonates, bicarbonates, hydroxides, and some other alkaline compounds. Eoff

(1918) obtained a patent on glycerol production by a yeast fermentation in an alkaline medium. He claimed that about 20 to 23 percent of the fermentable sugar was converted into glycerol. He listed as other products acetic acid, ethanol, and acetone. The organisms that he used were pure cultures of yeasts. He studied strains of both S. cerevisiae and S. ellipsoideus. He claimed best results by the use of S. ellipsoideus, variety "Steinberg", known as a California wine yeast. Optimum temperature was stated to be about 37° centigrade. Large scale fermentations were run by building up inocula from smaller ones, causing each successive culture to be grown in a medium made slightly alkaline by the reagent to be used for alkalizing the large fermentation. This procedure amounted to an acclimatization of the organism to the alkaline fermentation conditions. The alkali was added from time to time. It was recommended that the degree of alkalinity be maintained at a point just short of the point of inhibition of the culture growth. It was mentioned that sodium, potassium, and calcium carbonates, phosphates, and hydroxides may be used; sodium carbonate was preferred because of its cheapness and availability. A typical medium was made from molasses and contained about 11 percent total sugar. About 20 percent of the sugar was converted to glycerol.

Eoff (1919) stated that the glycerol yield was proportional to the amount of alkali used up to the endurance limit of the organism. He did not mention pH as being the controlling factor.

He showed that the optimum temperature was 30^0 to 32^0 centigrade in contrast to 37^0 as he had given previously in his patent. The best sugar concentration was stated to be 17.5 to 20 grams per 100 cc. of medium. In his molasses fermentations the sugar was calculated by the Fehling reducing sugar method. It was shown that the actual fermentable sugar was only 92 percent of the Fehling value. The corrected glycerol yields were then 19.6 to 27.1 percent on the basis of fermentable sugar. For each 100 parts of Cuban molasses used (47.8 percent as invert sugar) there would be produced:

Glycerol	10.04	parts
Ethanol	17.21	(removed by
Acetic acid, etc.	3.34	distillation)
Ash	8.00	
Sodium carbonate	15.94	
Organic non-sugars	22.20	
	76.73	parts

It may be noticed that in the resulting medium after alcohol removal, for every ten parts of glycerol there were 49.5 parts of non-glycerol solids. Originally, only about 50 percent of the glycerol present was actually recovered by direct vacuum distillation. However, treatment with ferrous sulfate followed by the use of hydrated lime powder caused a rather large part of the organic non-sugars to be precipitated. Evaporation to 30 percent glycerol, followed by steam distillation under vacuum, brought the glycerol recovery up to about 85 to 90 percent. Such a clarification method is rather costly, as are some other methods. The procedure has, however, emergency value.

2. Other processes

Connstein and Lüdecke (1924) described certain other types of alkaline fermentations. Most of these data may be found in the monograph of Lawrie (1928). Information on pH was not included, unfortunately.

McDermott , in a note for Lawrie's monograph (1928), explained his views on the molasses fermentation in the presence of alkali to form glycerol. He stated that ordinarily from pH 3.5 to 6.8 the Gay-Lussac reaction, or Neuberg's first equation, predominated. When the pH ranged from 7 to 8.8, a great increase in glycerol formation was noted in accordance with Neuberg's third equation. He claimed that the change of the hydrogen-ion concentration on the addition of the alkali caused a change in the reaction of the yeast enzymes on the carbohydrate being fermented. He noted that there was an indication in the literature that high salt or sugar concentrations alone enhance the production of glycerol. McDermott then mentioned that the main advantage of the use of a molasses medium over other media for alkaline fermentations is that the molasses acts as a buffer for the added alkali. The implication was that the better the buffer system, the better the glycerol yield. Maintenance of constant pH was stressed. Experimental data were given to show that molasses mash buffered the pH of the medium when sodium carbonate was added intermittently much better than did "synthetic" mash. The pH of both media averaged about eight, though the pH of the molasses medium was much more constant in its

value. The glycerol yields for the molasses and "synthetic" media were 18.54 percent and 15.24 percent respectively.

The question might arise as to whether the increase in the glycerol yield was caused by the constancy of the pH or by the higher concentration of solids in the molasses mash. The effect of bio-colloids as mentioned by Owen (1937) might come into effect in the molasses mash.

Neuberg and Kobel (1930) made studies on the decomposition of non-phosphorylated sugar by yeast with the formation of glycerol and pyruvic acid. Kobel (1931) made studies on the formation of equi-molecular amounts of glycerol and of pyruvic acid in cell-free fermentations of glucose. The effects of phosphates and other ions were studied.

Carothers, Hill, and Van Natta (1934) of E. I. du Pont de Nemours and Company, obtained a United States patent on fermentation glycerol wherein the sugar was fermented in an alkaline medium; the glycerol formed was distilled from the fermented product, and this glycerol was made alkaline with lime. The resulting mixture was blown with air in order to destroy phenols which were present.

Hesse (1935) noted that the maximum rate of fermentation to aldehyde of an 8.55 percent sugar solution in the presence of 4.27 percent sodium bicarbonate, was reached in 2.5 hours, which was 40 to 70 times greater than the usual fermentation rate. The acetaldehyde produced was changed to alcohol and acetic acid.

Equal parts of glycerol and acetic acid were formed.

The Norddeutsche Hefeindustrie, A.-G. (1938) described a method wherein the acids formed during a fermentation were neutralized by magnesium carbonate.

A patent of possible importance was granted to Krug and McDermott (1935). It was concerned with the use of ammonium hydroxide to control the alkalinity of fermentation mashes in order to produce glycerol. An important fact is that the ammonium hydroxide is not a "fixed" reagent, in that it is distillable itself, and the ammonium carbonate or bicarbonate that no doubt form are also not "fixed" and may be removed on distillation, as the ammonium carbonate is quite thermolabile, even in solution.

The purposes of this patent of Krug and McDermott are very closely aligned with some of the purposes of this thesis. They stated with regard to the Eoff (1918) method that "while such soda ash process produces a very good yield of glycerol, --- the addition of large amounts of soda ash and the like introduces difficult problems in the recovery and purification of glycerol, particularly when blackstrap molasses constitutes the source of the sugar."

An extended quotation from the patent of Krug and McDermott (1935), who are assignors to E. I. du Pont de Nemours and Company, should lend considerable weight to the importance of the work concerning glycerol production by means of fermentation.

The quotation follows:

A blackstrap molasses may have approximately the following composition:

	Percent
Fermentable sugars	40-48
Unfermentable sugars	5- 8
Proteins (N x 6.25)	4- 5
Gums and organic matters other than sugar, protein, or acids	15-18
Organic acids (as lactic)	1- 1.5
Ash	5- 8
Water	20-22

When a molasses of this type, such for instance as one containing 45 percent of fermentable sugar, 5 percent of unfermentable sugar and 6 percent of ash, is diluted with about three times its volume of water and fermented in the presence of sodium carbonate, in accordance with the disclosure of Eoff, in an amount of about 4.5 percent by weight of the mash or 28 percent of the weight of total sugar in the mash, each gallon of molasses would yield about 1.25 pounds of glycerol. This glycerol would be admixed with about 0.72 pounds of ash, originally present in the molasses, and about 1.67 pounds of the soda ash added in the process which would give the final ratio of glycerol to ash of about 1:1.91. These ash constituents interfere with the recovery of the glycerol by raising the boiling point of the solution. These salts further tend to accelerate polymerization and decomposition of the glycerol during the distillation thereof, thereby decreasing the yield. This is illustrated in the article by A. C. Langmuir in Ind. and Eng. Chem. of April, 1932, pages 378-380 inclusive. These salts further tend to cause decomposition of the non-glycerol organic matter present in the mash producing difficultly removable impurities in the glycerol, which result is favored by strongly alkaline reaction of the material from which the glycerol must be recovered.

An object of this invention is to provide a new process for the production of glycerol. A further object is to provide such a process which will not have the obvious above-mentioned disadvantages of the prior processes. A still further object is to provide a more economic process for the production of glycerol. Another object is to provide a process of producing glycerol in such a manner that the glycerol may be more readily and completely recovered and purified. Other objects will appear from a consideration of the following description of our invention.

In their patent various sugar solutions, and especially those made from molasses, were fermented with the alkalinity controlled

by the addition of ammonium hydroxide. Good glycerol yields were secured. It was mentioned that up to 1932 it was thought by those skilled in the art that "free ammonia" could not be used in place of soda ash with "commercially satisfactory results".

A main mash was prepared and inoculated with a strain of yeast. In about five to eight hours the fermentation became active, at which time ammonium hydroxide was added in such a quantity as to make the fermenting medium slightly alkaline. Fermentation was then allowed to proceed until the mash had become neutral or slightly acid whereupon more ammonium hydroxide was again added to slight alkalinity. This procedure was repeated as often as was necessary to keep the medium at the desired alkalinity. Various yeasts were used, but yeast "No. 16" of McDermott was preferred. Either ammonia gas or ammonium hydroxide solutions could be employed. In the examples cited, commercial ammonium hydroxide solution containing 26 to 28 percent ammonia was used. The incubation temperature was about 30° centigrade.

About 0.75 gram of ammonium sulfate was added per liter of molasses medium containing about 16 to 17 grams of invert sugar (calculated) per 100 cc. of medium. About 0.3 grams of secondary ammonium phosphate was added per liter of medium in some cases. The time of fermentation was about 72 to 84 hours. The pH values described usually oscillated between about 6.8 to about 7.3. It was mentioned in an example that a pH of 7.2 to 7.4 was produced

following an ammonium hydroxide addition after which "fermentation then ceases for a short time." When the fermentation "revived" in about two to three hours the pH was found to have dropped to about 6.8 to 7.0. The pH was measured colorimetrically. The sugar fermented was mentioned to have been 90.09 percent in one case and 92.16 percent in another. It was stated that "after fermentation the mash will contain from about 6.0-6.5 percent by volume of alcohol and about 2.70-3.15 grams of glycerol per 100 cc. This indicates a yield on the total sugar (3950 pounds) in the molasses of 15.8-18.5 percent glycerol and 27.9-30.5 percent alcohol."

The alcohol was removed in the usual manner and from 20 to 40 percent of the ammonia was recovered by a suitable scrubber in the alcohol vapor line. Vacuum evaporation concentrated the dealcoholized medium to a heavy syrup. The glycerol was recovered from this syrup by the use of a spray tower such as that indicated by Lawrie (1928). However, such a tower is not entirely necessary since the low salt concentration allows the use of the more usual types of distilling equipment.

Acetic acid was formed during the fermentation and was recovered by the usual methods.

In the examples of this patent, it was shown that the ratio of glycerol to ash was about 1.0 to 0.7 as compared to the corresponding ratio of 1:1.91 for the Neff method. This was "a reduction of the ash with respect to the glycerol to about 40 percent of that

which would be obtained with the soda ash process." The handling of ammonia was simpler than the handling of strong caustics, and the amount of ammonia required constituted a noticeable economy. Recovery was much simpler and more feasible.

It is well to note that in this patent Krug and McDermott claimed maintenance of pH from 7 to 8 although the descriptions hardly mention any pH value more alkaline than about 7.3 or 7.4. Such a value was not always maintained but was reached intermittently upon the addition of the ammonium hydroxide, after which the pH sometimes fell to 7 or below. It appeared that perhaps time intervals of fermentation in the acid range may have been necessary for a successful fermentation when ammonium hydroxide was used to control the pH. Vokorny (1913) noted a toxic action of ammonia on yeast.

The use of ammonium hydroxide required the formation of ammonium carbonate or ammonium bicarbonate or both during the fermentation.

TABLE 6

AMMONIUM HYDROXIDE ADDITIONS WITH RESPECT TO TIME IN ORDER TO KEEP pH ABOUT SEVEN IN A JAVAN MOLASSES FERMENTATION OF THREE LITERS

Dose number	Volume cc.	Hours after seeding
1	20	5:10
2	15	10:55
3	10	14:05
4	5	16:40

(From Krug and McDermott (1935))

Both salts were derived from a weak base and a very weak acid. The presence of such salts had the advantage of buffer effect in the medium. The volumes of ammonium hydroxide solution (containing 28 percent ammonia) added to three liters of a Javan cane molasses medium containing 18 grams of invert sugar (calculated) per 100 cc. of medium are noted in Table 6.

A further discussion of the ammonium hydroxide fermentation method will be found in a later section of this thesis.

IV. EXPERIMENTAL

A. Apparatus

1. pH Measuring Instruments

a. Cameron pH Meter. The pH meter used was a Cameron Meter of the double scale type which will give direct readings in either millivolts or pH. It was made by the Eisendrath Memorial Laboratories of Racine, Wisconsin, and was obtained by this laboratory from the Wilkens-Anderson Company of Chicago, Illinois. It is a glass electrode meter which operates entirely from batteries and is non-portable inasmuch as a storage battery is necessary. Its operation was most satisfactory when operating directions were followed carefully. It was advisable to keep all solutions used in connection with the pH measurements at the same temperature as nearly as possible to eliminate any errors arising from temperature differences of the opposing half-cells. Inasmuch as the instrument is widely used in industry and other scientific fields, it will not be described further as desired information may be obtained from the manufacturers or from the distributors.

b. Coleman pH Meter. This meter is similar to the Cameron Meter in some respects, but it is different in that it is portable. It has the sample cup contained in the meter box, while the

Cameron meter has a separate detector unit allowing easier access to the sample cup and simpler manipulation. However, either instrument is quite satisfactory for pH measurements where a glass electrode measurement is desired. This instrument is made by the Coleman Electric Company of Maywood, Illinois. It is a ballistically operated instrument, while the Cameron Meter is not, the balance point of the latter being a null point on a galvanometer. A pH measurement may be made with either instrument in a matter of seconds.

2. pH Control and Recording Instrument

Inasmuch as it is difficult to control pH by means of buffers at all desired levels, a means of very accurate control was desired. A permanent record of the pH against time was also very desirable. Buffers may keep the pH of a medium at approximately the pH sought, perhaps within 0.5 or 1 pH unit. Sometimes rather high salt concentrations are necessary for buffers to be satisfactory, but this salt may alter the process being studied to such an extent as to be unsatisfactory. It was desired in some cases to operate fermentations with certain reagents that could be easily removed at the completion of the fermentation. Some reagents are not satisfactory for buffers if the ease of salt removal is to be maintained. Such pH control agents are calcium hydroxide and ammonium hydroxide. Control of pH by sulfur dioxide in the presence of ammonium sulfite, calcium sulfite, or magnesium sulfite were also considered or used.

Considerable correspondence was made with various manufacturers and dealers of pH control instruments operating from a glass electrode. It was found that such instruments are quite new, complicated, and expensive. The price of such an instrument was found to be in the neighborhood of $1000. With this in mind some experiments were made with certain vacuum tube circuits to see if a control instrument might be built which would be simpler than the commercial instruments, as well as much cheaper. Shortly after this work had been begun, a commercial pH recorder was obtained which was adapted to pH control. Because of this acquisition, further work on the development of such an instrument was discontinued.

The equipment secured was a Cameron pH Recorder, obtained from the Wilkens-Anderson Company in the spring of 1939. It was set up and tested as a recorder of pH at first. Records were made of pH against time for yeast fermentations and for the butylacetonic fermentation. The curves obtained were excellent. The machine recorded the pH continuously for 24 hours with little attention except to occasionally check potentiometric balance. The record from 10 P.M. to 8 A.M. was especially convenient.

After it was found that the instrument operated satisfactorily, the circuit was adapted to control operation. A magnetically controlled Mercoid switch was installed on the instrument in such a way that a magnet moved in conjunction with the recording pen connected to the recording potentiometer screw. For the details of the operation of the recorder, one should see the operating directions that

came with the instrument; the circuits are also included. The details of the added circuits of the control mechanisms are shown below; they were developed to a large extent in these laboratories. No attempt is made here to describe the commercial instrument as it is exceedingly complex and is described by the manufacturers. Many of the references to parts and contact numbers and trip-wheel positions refer to the code used in the manufacturers diagrams.

The following diagrams of circuits and parts are concerned only with the control mechanism. The first circuit involves the simple magnetically operated Mercoid switch which came with the instrument. It is normally closed, but opens under magnetic influence. The magnet is usually behind the switch keeping it open, thus keeping the solenoid valve inoperative while the pH is at the desired level. The control circuit is shown in Figure 1.

It was found that this type of circuit was not entirely satisfactory since control was maintained only while the pH of the medium tended to decrease, i.e., while acids formed as time proceeded. However, toward the end of some fermentations, the medium became less acid, that is, the pH increased. The circuit was designed to add base as the pH decreased with the magnet proceeding to the right past S_2 of Figure 1. When the magnet goes to the right of S_2 a short distance, S_2 closes, thus operating the valve which allows the base to flow until the pH increases. The magnet then moves to the left, thus opening S_2 again and shutting off the solenoid valve. But when

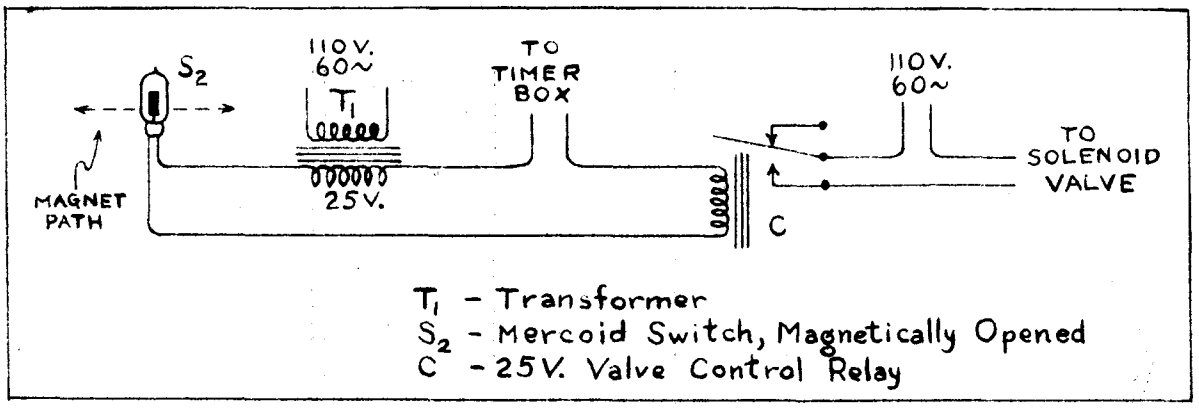
the pH increases from the controlled pH value, then the switch S_2 opens again causing the base to add as before, and the magnet goes farther and farther to the left. The base adds until the base reservoir is empty. Control of pH is lost, and the experiment is spoiled.

A safety circuit was designed to correct for this difficulty so that control of pH would be maintained as usual when the pH of the medium tends to decrease; but when it tends to increase, the base cannot be added; merely a pH record results. This modified circuit is shown in Figure 2.

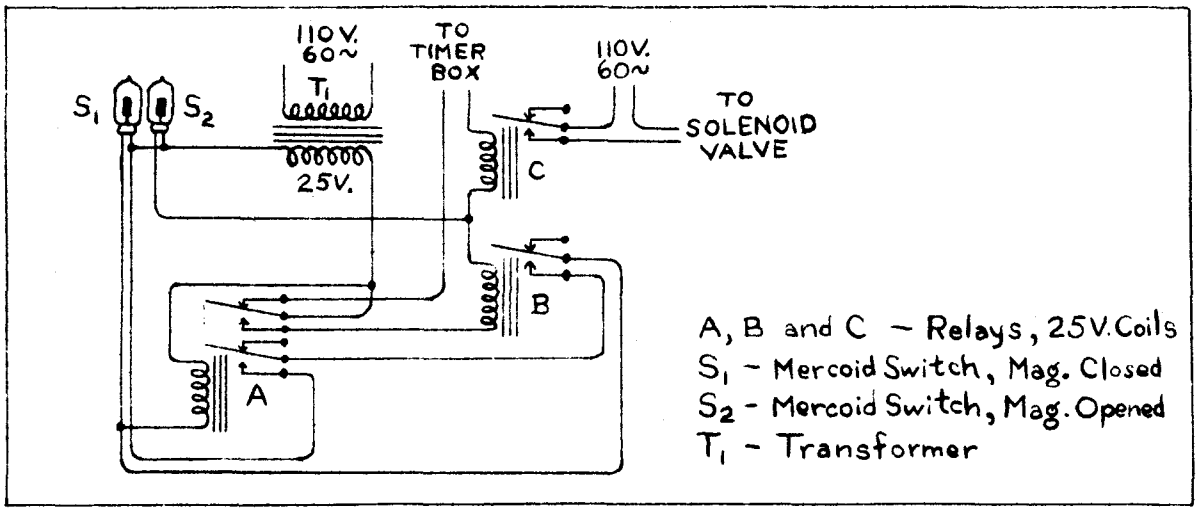
The magnet operates normally behind S_2 which came with the instrument; in this position the switch is "off" as noted previously in Figure 1. As the magnet proceeds to the right with decreasing pH, the switch S_2 closes thus operating relay C which then opens the solenoid valve. Base is added, and pH increases until S_2 opens again. But should the occasion arise that the medium becomes more basic than the value for which the control is set, the magnet will proceed to the left of S_2, thus causing the addition of some excess base for an instant. However, as S_1 is adjacent to S_2, the magnet quickly closes S_1 which operates relays A and B. Having been operated, these relays do not allow any more base to add since relay C is now open. Relays A, B, and C will now remain in this position regardless of what happens until sufficient acidity forms in the medium to bring the magnet back to the right to S_2, or until the

operator can observe the situation. If acid forms causing the magnet to move behind S_2 thus opening that contact, then all relays fall back to normal position, and normal operation will proceed. When the pH increases from the pH control value again instead of the reverse, then the procedure would be repeated. The operation of this safety circuit was quite successful. The closer S_1 and S_2 are together, the greater is the accuracy of control. For a fermentation that continually becomes more alkaline there is no control without revising the above circuit by reversing the positions of S_1 and S_2 and by placing acid in the reservoir instead of base. Since relays A and B keep themselves closed after they are operated, it is necessary not to jar them, as control will be lost if they are opened by any means other than by S_2.

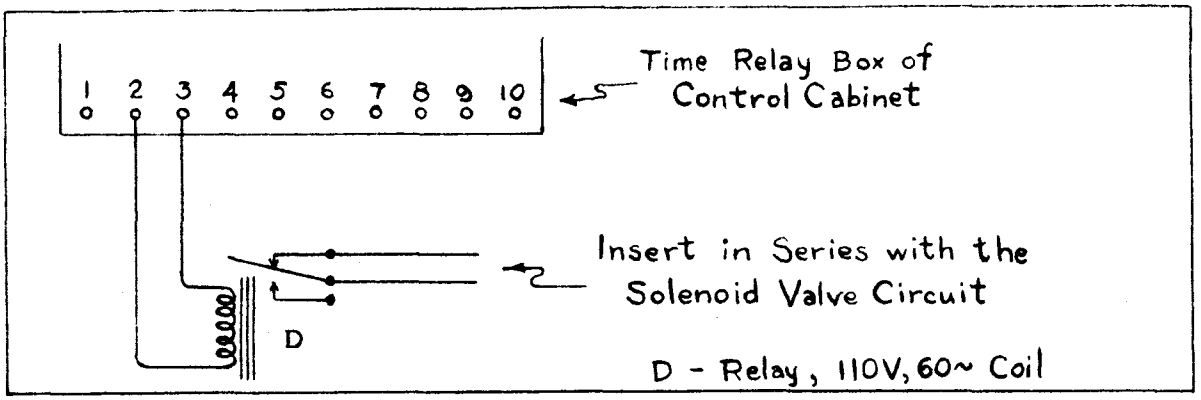
It was found after several operations that sometimes the balancing mechanism of the Recorder, which balances the potentiometers once every four minutes, commenced operation at the time that base was being added. When this is the case, base is added again when the timer trip-wheel is in position 1-4. This is because of previous design of the instrument incorporating an automatic sampling period during the four minute time cycle. Through the advice of Dr. Cameron, another relay was installed as shown in Figure 3 so that the solenoid valve can not operate when the trip-wheel is in position 1-4. The valve should be able to operate only during the recording cycle, or when the trip-wheel is in position 4.



INITIAL pH CONTROL CIRCUIT
Figure 1.



SAFETY CIRCUIT
Figure 2.

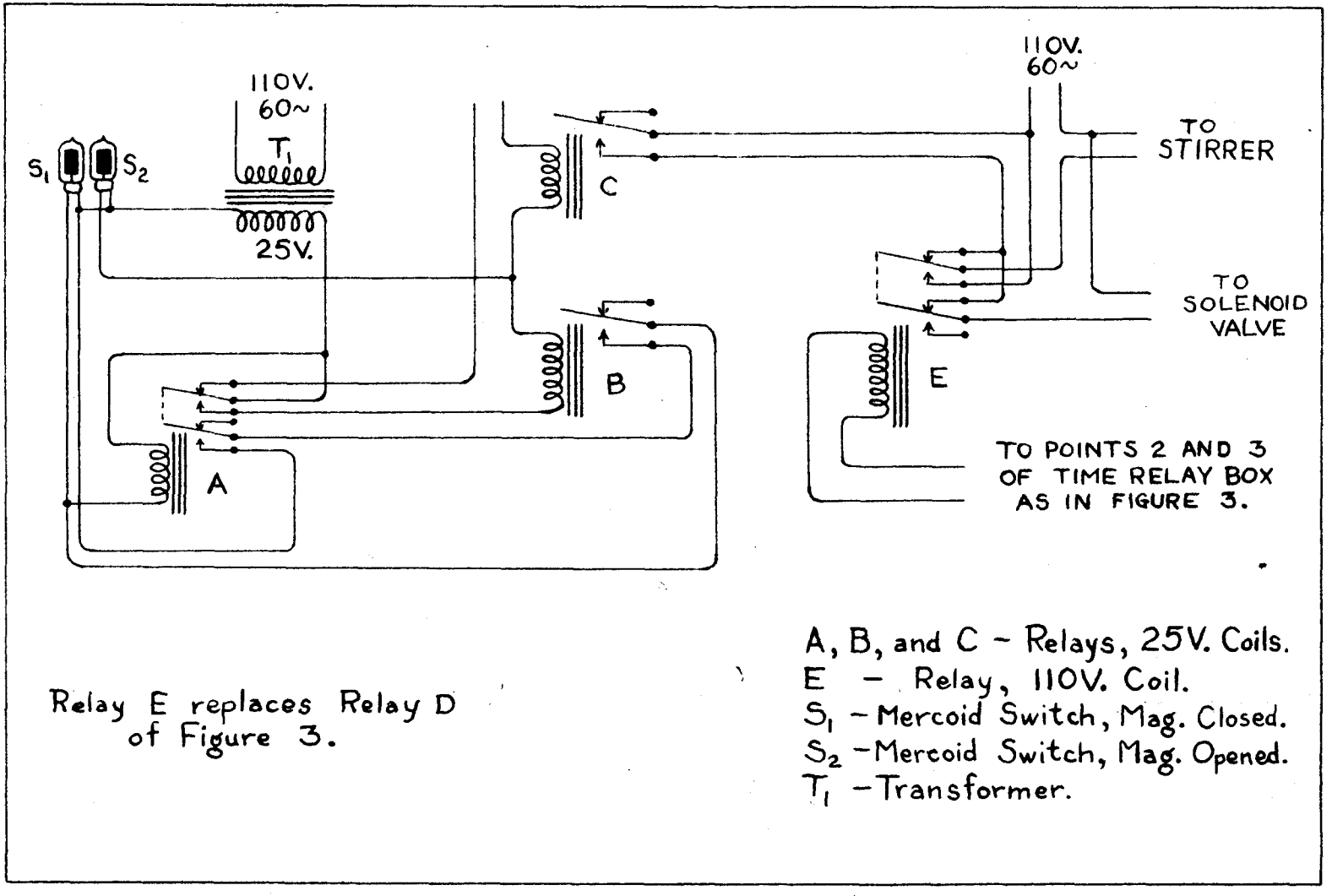


CIRCUIT ALLOWING BASE TO ADD ONLY IN THE RECORDING PORTION OF THE CYCLE
Figure 3.

It was found after the installation of the relay D as indicated in Figure 3 that operation of the entire control mechanism was satisfactory. The only difficulties encountered were difficulties such as were caused by an open condenser in the photoelectric amplifier circuit, an open circuit in a dry cell, air bubbles in the salt bridge, an electrical leak in a glass electrode, occasional low charge in the storage battery, and a few other situations. A relay spring broke on one occasion. In an instrument as complex as is this automatic pH Recorder-Controller, there are many possibilities for a mechanical defect to arise. Operation is successful, but the machine must be watched rather carefully in order to detect rapidly, if possible, the source of trouble if it arises.

It was decided to further complicate the already complex circuit by adding the necessary circuit for automatically stirring the fermenting medium for 15 seconds of every four minutes. The time control relay operated on the cycle which would allow this control to be adapted. The resulting entire control circuit is shown in Figure 4. The medium is stirred 15 seconds out of four minutes, and also during the time that the solenoid valve control is allowing the base to add to the medium. Each operation is independent of the other and there is no interference between circuits. The final circuit made use of the advantageous parts of previous circuits with an intermittent stirrer control added as shown in Figure 4.

The circuit diagramed in Figure 4 operated quite successfully.



COMBINED SAFETY, pH CONTROL, AND AUTOMATIC STIRRING CIRCUIT
Figure 4.

There were occasional mechanical difficulties such as the breaking of a spring of a relay and other such actions, but after a few controls the difficulties were minimized. The method seemed quite promising and several control operations have been run for six days continuously without any trouble whatsoever either with the machine or its adjuncts. Control was maintained with ease to within 0.1 pH unit of the desired value. Occasional checks by the pH meters verified the record of the control. The place at which control of the pH ceased was where the pH began to increase toward the end of some fermentations. However, this increase was usually very slight and gradual.

B. Methods of Procedure

1. Analytical methods

The analysis of the fermentation was designed to be carried out as economically as possible, and also with the greatest practical degree of speed and accuracy. The accuracy is, of course, of primary importance; however, where large numbers of determinations are to be made, it is most desirable to be able to run these determinations en masse with accurate control of analytical conditions. One of the most common methods for the determination of reducing sugar is that of Shaffer and Hartmann (1921). The amounts of the necessary reagents for a single sugar determination are large thus making a single determination relatively expensive. The method also has the disadvantage of being rather inconvenient to use for large numbers of analyses simultaneously. Adjustments of copper reducing conditions must be very accurate in order to obtain reproducible results. Most glycerol analysis methods are very complex and tedious; they usually depend on the complete removal of sugars prior to actual analysis. In some methods there are days between the start and the finish of a single analysis. Even then, the accuracy of the determination is sometimes doubtful. Determinations of glycerol en masse in the presence of sugar by some of the methods would surely try the patience of even the most patient analyst. In many cases, it is very advanta-

geous to have the glycerol result within a few hours at most of the time the sample is taken, thus rendering the long analysis quite undesirable if another more rapid and accurate method is available.

a. Determination of sugar. There are numerous sugar analysis methods that may be found in most of the standard textbooks on food analysis and related subjects, but most of the methods are modifications of the ferricyanide reduction procedure of Hagedorn and Jensen (1923) or of the original gravimetric copper reduction method of Munsen and Walker (1906). The most commonly used modification of the Munsen and Walker method in this country is the volumetric method of Shaffer and Hartmann (1921).

The ferricyanide method depends on the reduction of potassium ferricyanide to ferrocyanide. Modifications of the Hagedorn and Jensen method have been made by Blish and Sandstedt (1933) and by Gore and Steele (1935). Objections to this method have been pointed out by Shaffer and Somogyi (1933) and by Somogyi (1937). A main objection was the lack of specificity to sugar in contrast to the specificity shown by copper reduction methods. The method of Shaffer and Hartmann (1921) is a macro-determination, being run in 300 ml. Erlenmeyer flasks. It was quite inconvenient to run large numbers of determinations in a short time by this method because of the exact control necessary during the copper reduction by the sugar. Improvements in the analytical methods were made by Stiles, Peterson, and Fred (1926) and later, as was mentioned previously, by Shaffer and

Somogyi (1933), and still later by Somogyi (1937). The method of Somogyi (1937) was employed as the basis for development of the analytical methods used during this research. A modified method described in the thesis of Guymon (1939) was also used.

The composition of the reducing sugar reagent recommended by Somogyi (1937) for determinations of micro quantities of sugar in blood is given in Table 7.

TABLE 7

SOMOGYI REAGENT FOR THE COPPER-IODOMETRIC DETERMINATION OF VERY SMALL AMOUNTS OF SUGAR

Reagent	Quantity
Na_2CO_3 (anhydrous)	25 gm.
Rochelle salt	25 gm.
$CuSo_4 \cdot 5H_2O$	4 gm.
$NaHCO_3$	20 gm.
Na_2SO_4 (anhydrous, analytical grade)	200 gm.
KI	1.5 gm.
KIO_3	6.0 cc. of 1 N. solution
Water	To make 1 liter of solution

According to Somogyi (1937) the sodium sulfate has the effect of stabilizing the solution, eliminating self-reduction of the reagent. It also depresses the carbonate ionization, and thus the alkalinity of the solution. The method of preparing of the reagent is quoted from the paper of Somogyi:

> Preparation - The carbonate and Rochelle salt are dissolved in about 800 cc. of water; then 40 cc. of a 10 percent $CuSO_4$

solution are introduced with stirring. This is followed by the addition of the bicarbonate, sulfate, and iodide. The solution is heated to boiling, kept boiling for about thirty seconds, cooled, and after the addition of the KIO_3 diluted to 1 liter.

Since the chemicals, even though of analytical grade, contain solid impurities, the reagent requires filtration. Upon standing for a day or two before filtration, the impurities settle out as flocculent particles and can be removed more completely than if the solution is filtered fresh.

It should be noted that filtration of the above reagent should not be attempted through filter paper or other cellulosic materials since there is some hydrolysis to reducing sugars which will naturally affect subsequent sugar determinations by means of the reagent. Cuprous oxide will gradually settle out on prolonged standing. The presence of finely divided particles in the reagent induces reoxidation of cuprous oxide. These effects are discussed by Somogyi (1937) and by Benedict (1931).

Procedure: The reagents used for the micro sugar determinations were:

(A) The copper-iodometric reagent.

(B) $KI-K_2C_2O_4$ reagent which contains 2.5 percent of each in water.

(C) 5 Normal H_2SO_4 solution (Keep away from rubber!!).

(D) $Na_2S_2O_3$ standard solution approximately 0.005 normal.

The reaction was run in 22 x 250 mm. Pyrex test tubes at first. The procedure employed was as follows: The sugar sample to be determined is clarified with basic lead acetate and an aliquot is added to the test tube such that the solution added shall contain from

0.05 to 0.5 mg. of dextrose or its equivalent in a volume of 5.0 ml. To the 5.0 ml. of sugar solution in the test tube is added 5.0 ml. of reagent (A). After mixing, the tube is placed in a boiling water bath for exactly 20 minutes as recommended by Somogyi (1937), after which it is placed in water to cool the contents to 30^0 centigrade. Then 2 ml. of (B) is added, followed by 1.0 ml. of solution (C). The resulting solution is thoroughly mixed and allowed to stand for about five minutes at 30^0 with occasional shaking. The mixture is then titrated with standard sodium thiosulfate solution (D) with about six drops of starch solution used as an indicator. A 5 or 10 ml. micro-burette is advisable for the thiosulfate. The volume of thiosulfate should be estimated to the nearest 0.01 ml. A blank should be run with all sugar determinations. Preferably two blanks should be run at the same time that the sugar is determined, and the sugar determinations should be run in duplicate. It was found after a number of determinations that a linear equation could be used for calculating sugar from the thiosulfate titration value. Equation (1) was fairly satisfactory for the data obtained.

$$\underline{D} = 0.116\ \underline{V} + 0.01.$$

In the equation, $\underline{D}$ is expressed as milligrams of dextrose and $\underline{V}$ is the volume of thiosulfate in milliliters obtained by subtracting the experimental titration value from the blank titration value, and converting this volume to milliliters of 0.005 normal thiosulfate if the reagent is not of that normality.

It may be noted that V is directly proportional to the quantity, D - 0.01, and it is directly proportional to the weight of cuprous oxide precipitated which is proportional to the sugar. V is not the thiosulfate titration value.

Typical results are given in Table 8. The dextrose used for the standardization was Pfanstiehl's C.P. anhydrous dextrose. It was dried one hour at 110° centigrade and the rotation was measured. The sugar was satisfactory. A standard solution was made to contain

TABLE 8

STANDARDIZATION OF THE SOMOGYI SUGAR METHOD AGAINST PURE DEXTROSE

Milligrams Dextrose Present	Volume, V of 0.005 N. thiosulfate	Milligrams Dextrose Found	Milligrams Dextrose Present	Volume, V of 0.005 N. thiosulfate	Milligrams Dextrose Found
0.050	0.16	0.02	0.200	1.74	0.212
0.050	0.08	0.01	0.300	2.57	0.308
0.050	0.34	0.049	0.300	2.28	0.274
0.100	0.82	0.105	0.300	2.44	0.293
0.100	0.79	0.101	0.400	3.38	0.402
0.100	0.82	0.105	0.400	3.36	0.400
0.100	0.73	0.095	0.400	3.50	0.416
0.200	1.69	0.206	0.500	4.32	0.510
0.200	1.74	0.212	0.500	4.16	0.492
0.200	1.53	0.188	0.500	4.24	0.502
0.200	1.50	0.184	-	-	-

0.1 mg. of dextrose per ml. of solution. Volumes were measured from a 5 ml. burette. No solution was used which was over 24 hours old, since the growth of molds generally alters the solutions by about

that time.

The analyses, as indicated in Table 8, were not all that could be desired. There appeared to be an evaporation during the heating, thus changing the concentration of reagents, and also the presence of oxygen of the air might cause reoxidation of the cuprous oxide. Hence, it was decided to modify the above procedure by covering the test tubes with glass bulbs during heating and previous to titration. It was thought that lengthening the time of heating might also be advantageous. After some preliminary tests it was decided to make the following two modifications to the procedure described above:

(1) The time of heating in the water bath is increased from 20 minutes to 35 minutes.

(2) The reaction tubes are covered with glass bulbs at all times up to the time of titration with sodium thiosulfate solution.

It was necessary to determine a new equation for sugar determinations by the titrations with the modified method. Equation (2) is the new equation. The definition of terms is the same as for Equation 1. It may be noted that the constant 0.001 is nearer zero than was the similar constant of Equation 1.

$$(2) \quad \underline{R} = 0.1071\ \underline{V} + 0.001$$

The reagent "B" ($KI-K_2C_2O_4$) should be added so that it remains on the surface of the mixture in order that iodine as it is formed will have to go through potassium iodide in order to escape and

will be dissolved thus preventing escape while effervescence proceeds. Typical results appear in Table 9.

TABLE 9

DEXTROSE ANALYSES BY THE MODIFIED SOMOGYI COPPER-IODOMETRIC METHOD

Milligrams Dextrose Present	Volume, ml of 0.005 N. thiosulfate	Milligrams Dextrose Found	Milligrams Dextrose Present	Volume, ml of 0.005 N. thiosulfate	Milligrams Dextrose Found
0.030	0.28	0.030	0.300	2.79	0.300
0.050	0.47	0.051	0.400	3.72	0.400
0.100	0.93	0.101	0.400	3.72	0.400
0.200	1.84	0.198	0.400	3.73	0.401
0.200	1.88	0.202	0.500	4.57	0.492
0.300	2.79	0.300	0.500	4.56	0.491

The results of Table 9 indicate that the modified sugar method is quite satisfactory provided that the quantity of sugar in the sample does not greatly exceed 0.4 mg. It may be noted that the low values were found fairly accurately; however the percentage error with these small amounts is great. The main objection to this method is that a high dilution is necessary for many fermentation analyses.

The determination of sugar has also been considered by other investigators of these laboratories. In order to eliminate the high dilutions required for sugar determinations such as were necessary in the previous methods described, and to render the reagent and results more reproducible, Guymon (1939) developed what is called

reagent "G" for sugar determinations where dilution requirements are not so high. Reproduction of results was quite satisfactory when subsequent reagents were made up in the same way. It was found to be advisable to make the pH of the reagent the important factor rather than the actual amounts of basic reagents employed since the use of different batches of chemicals may produce a different pH in the final reagent. Reagent "G" of Guymon is given in Table 10.

TABLE 10

COPPER-IODOMETRIC REAGENT "G" FOR THE DETERMINATION OF SUGAR ON A SEMI-MICRO SCALE

Reagent	Quantity
Na_2CO_3 (anhydrous)	53 gm.
NaOH	About 11.4 gm. saturated solution is added to pH 9.48; this requires about 7.5 ml.
$NaKC_4H_4O_6 \cdot 4H_2O$ (Rochelle salt)	125 gm.
$CuSO_4 \cdot 5H_2O$ (add as H_2O solution)	37.5 gm.
KI	1.0 gm.
KIO_3	3.567 gm. (exact amount).
Na_2SO_4 (anhydrous)	50.0 gm.
Water	to 1 liter final volume.

The procedure of making up the reagent is as follows: The sodium carbonate and Rochelle salt are dissolved in approximately 300 ml. of distilled water after which the copper sulfate is added as an aqueous solution with stirring so that carbon dioxide will not be evolved. The use of a funnel with its exit under the surface of the carbonate solution is quite helpful. After this the potassium

iodide and sodium sulfate are added. The solution will now have a pH of about 9.1 as measured by a glass electrode pH meter. The sodium error is not considered and would not be important so long as there is a constant deviation. Water is added to bring the total volume to about 950 ml. The sodium hydroxide solution is now added little by little with stirring until a pH of 9.48 is obtained as measured by a glass electrode system. If the pH is from 9.4 to 9.5 the results will be reasonably satisfactory.

The solution is heated to boiling and the boiling is continued for about three to five minutes. The solution is then cooled, the iodate is added, and water is added to make the final volume one liter. Considerable cuprous oxide usually forms. The solution should be allowed to stand for several days, or perhaps a week, and it should be kept in a Pyrex bottle or flask. At the end of this time, the reagent may be filtered through asbestos or other inert material. Filter paper should not be used as it hydrolyzes somewhat under the influence of the alkaline solution. As an alternative, the clear supernatant liquid may be siphoned from the precipitate if suitable precautions are taken against agitation of the precipitate. Any suspended precipitate in the reagent is very undesirable, and errors will be noted in the results, especially if the precipitate is cuprous oxide.

The reagents for the sugar determination by this method are similar in some ways to the reagents of Somogyi (1937) but they

result in about ten times the oxidizing capacity. The reagents to be used are:

(1) Reagent "G".

(2) Solution containing 12.5 gm. KI and 25 gm. $K_2C_2O_4 \cdot H_2O$ per 100 ml. of distilled water.

(3) Solution 7.5 normal in H_2SO_4 (Keep away from rubber!!)

(4) Sodium thiosulfate solution about 0.05 normal.

(5) Starch solution in saturated aqueous NaCl solution.

The method of procedure is similar to the method previously described. The test tubes used are smaller (25 x 150 mm.), although the actual size is not critical. To 5 ml. of reagent "G" is added 5 ml. of clarified sugar sample containing not over 10 mg. of sugar. Mix thoroughly and heat covered in a boiling water bath for 30 minutes; then cool to 30^0 centigrade. Add 2 ml. of the iodide-oxalate reagent (2), and then add the sulfuric acid very cautiously until all the iodine that will form is liberated. Slightly over 1 ml. is necessary. Finally titrate with the thiosulfate solution using the starch indicator for the endpoint.

Analyses are most satisfactory when the sugar in the sample is between 1 and 10 mg. In some fermented media practically no dilution is necessary for a determination. Clarification is recommended, however. Basic lead acetate or alumina cream are usually used, but other agents are applicable depending on the medium. Doak (1939) described a new method utilizing cadmium hydroxide for plant extract

treatments. In analyses, Equation (3) was found to hold quite well for the reagent and technique used.

$$(3)\quad \underline{D} = 1.305\ \underline{V}$$

In this equation, $\underline{D}$ is expressed as milligrams of dextrose and $\underline{V}$ is the volume in ml. of 0.05 normal sodium thiosulfate solution which is obtained by subtracting the experimental titration value from the blank value as described on page 59. It is advisable to derive a new equation each week or so in order to be sure of the equation constant. It changes with decreasing rapidity as the solution ages, but the rate of change closely approaches zero after about a month of standing. The constant varies for different sugars. The method is considered entirely satisfactory. One must be very careful with the sulfuric acid reagent to keep it free of oxidizable materials such as become dissolved in the acid on long exposure to rubber. The unsaturated material extracted from rubber caused trouble until the source of the difficulty was discovered.

The modified method of Somogyi was used in the experimental work until the method of Guymon (1939) became available. Both methods were satisfactory; however, the method of Guymon was more convenient for sugar determinations in the concentrations generally encountered in this work.

b. Determination of ethanol. The ethanol determination was based on the standard distillation - density method of the Association of

Official Agricultural Chemists (1925). Usually about 300 ml. of medium were distilled in the presence of calcium carbonate in order to keep volatile acids from distilling. Kjeldahl distillation apparatus was used, and 100 ml. of distillate were collected in a 100 ml. volumetric flask. The density of this solution was measured at 25° centigrade by means of a chainomatic Westphal balance. Standard conversion tables were employed to obtain the grams of ethanol per 100 ml. of distillate.

In cases where ammonia might distil with the ethanol and water, a second distillation was necessary, sulfuric acid being added before the distillation until an acid reaction to a non-alcoholic indicator was noted. Further analytical directions have been given by Gehle (1922).

c. Determination of sulfite. Sulfite was determined by titration with iodine solution as described by Kolthoff and Sandell (1937), and Kolthoff (1929). Free sulfite was first titrated in acid solution. Then on addition of sodium bicarbonate, the bisulfite-aldehyde complex decomposed liberating the combined sulfite which was titrated with iodine solution. This determination is closely related to the acetaldehyde determination.

d. Determination of acetaldehyde. The acetaldehyde may be estimated by an iodimetric determination of the difference between total sulfite and fixed sulfite in the medium. The acetaldehyde fixes the

bisulfite in a quantity proportional to the quantity of acetaldehyde present. The fixed sulfite is liberated by alkalization of the solution. Detailed directions may be found in the papers of Tomoda (1929) and Jaulmes and Espezel (1935). A note is also given in the monograph of Lawrie (1928). Other methods were described by Gehle (1922).

g. Determination of glycerol. The glycerol was determined by the method of Fulmer, Hickey and Underkofler (1940). This method consists of a determination by a copper reduction method followed by an oxidation of both the sugar and the glycerol by 0.1 normal ceric sulfate solution. This analytical method was developed especially for these studies. Further references may be found in the paper mentioned. Numerous other analytical methods are given in the monograph of Lawrie (1928) and in the paper of Gehle (1922).

2. Development of an optimum semi-synthetic medium.

It was desired to have a medium with which to study the glycerol fermentation which would be as nearly synthetic as possible, and also would have a minimum amount of "fixed salts". The purpose of using such a medium was to make the analysis of desired products, such as glycerol, simpler and more expedient, in order to follow the desired investigations with more ease. Such a medium would be used only to develop or improve methods for glycerol production.

Dextrose was used for the preliminary medium in order to have as

few analytical complications as possible, such as inversion of sucrose before reducing sugar determinations can be made. Yeast extract (Difco anhydrous) was selected as the only required material which was not a pure chemical of known composition as it is one of the most concentrated yeast nutrient sources; thus, very little should be required.

The work of Fulmer, Nelson and Sherwood (1921) and information given by Buchanan and Fulmer (1930b) were considered preceding the experimental work. The term, "optimum medium", is intended to indicate optimum with respect to alcohol yield, minimum salts, and speed of reaction rather than to yeast growth. The optimum medium was prepared for the normal alcoholic fermentation.

The composition of Table 11 was assumed for an initial basal medium.

TABLE 11

THE BASAL MEDIUM

Reagent	Weight per 100 ml. of Medium	
Dextrose (anhydrous)	15	gm.
Yeast extract (Difco anh.)	0.5	gm.
NH_4Cl	0.188	gm.
KH_2PO_4	0.05	gm.
$K_2HPO_4 \cdot 3H_2O$	0.05	gm.
$MgSO_4 \cdot 7H_2O$	0.01	gm.
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.01	gm.
$CaCl_2$	0.01	gm.

The original medium employed for carrying the yeast culture

contained 5 grams of dextrose and 0.5 grams of yeast extract per 100 ml. of medium. Tap water was used. Sterilization was at 10 lbs. steam pressure for one-half hour. Culture flasks were 125 ml. Erlenmeyer flasks containing 70 ml. of the dextrose-yeast extract medium. The pH of the medium was adjusted to 5 before sterilization.

A medium containing 5 grams of sugar in 100 ml. of solution is called a 5 percent medium. Other media are described similarly for convenience. The basal medium was thus a 15 percent medium. Inocula for the flask series were 10 percent media, as near optimum with regard to materials other than sugar as were found from time to time so that the inocula would alter the composition of the media being studied as little as possible. A 5 percent inoculation means that 5 ml. of inoculum is added per 100 ml. of medium.

A series of media was fermented first keeping all ingredients constant except the yeast extract which was varied in order to find the least amount of this agent which could be used and still obtain a maximum of alcohol. The series is described in Table 12.

By observation of Table 12, it is evident that the optimum concentration of yeast extract is about 0.7 grams in 200 ml. of medium, or 0.35 grams per 100 ml. Accompanying analyses were made for residual sugar by the method of Somogyi (1937). Yeast extract was found to have negligible reducing effect on the copper complex. The flasks containing 0.7 grams and over of yeast extract showed about 1 percent or less of residual dextrose. The other flasks contained more than

1 percent of residual sugar. It was estimated that the desirable amount of yeast extract should be about 0.375 grams per 100 ml. of medium. This value was used for subsequent investigations.

TABLE 12

EFFECT OF YEAST EXTRACT CONCENTRATION ON ETHANOL YIELD

Flasks: 200 ml. medium in 500 ml. Erlenmeyer flasks.
Medium: 15% dextrose medium shown in Table 11, except for yeast extract. pH = 5.
Sterilization: 10 lbs. for 30 min.
Inoculum: 10% dextrose medium, 10 ml. to each flask. (5% inoculation)
Incubation: 5 days at 30° C.

Grams of Yeast Ext. in 200 ml.	Density of distillate d_{25}^{25}	Grams of Ethanol, Total
0.0	0.9925	4.13
0.0	0.9924	4.19
0.1	0.9900	5.60
0.1	0.9900	5.60
0.2	0.9863	7.89
0.2	0.9868	7.58
0.3	0.9846	8.95
0.3	0.9843	9.15
0.4	0.9817	10.85
0.4	0.9824	10.39
0.5	0.9798	12.12
0.5	0.9796	12.26
0.6	0.9796	12.26
0.6	0.9791	12.60
0.7	0.9786	12.94
0.7	0.9789	12.73
0.8	0.9793	12.46
0.8	0.9791	12.60
1.0	0.9792	12.53

Having determined the optimum yeast extract concentration, the next material taken for study was ammonium chloride. A series of flasks was prepared in a manner analogous to the series of Table 12. The data obtained are given in Table 13.

TABLE 13.

EFFECT OF AMMONIUM CHLORIDE CONCENTRATION ON ETHANOL YIELD

Flasks: 200 ml. in 500 ml. Erlenmeyer flasks.
Medium: 15% dextrose, 0.375% yeast extract; other materials as in Table 11, except NH_4Cl. pH = 5.
Sterilisation: 13 lbs. for 20 minutes.
Inoculum: 10% dextrose medium, 5% inoculation.
Incubation: 4 days at 30° C.

Grams of NH_4Cl in 200 ml.	Density of distillate, d_{25}^{25}	Grams of Ethanol, Total
0.0	0.9801	11.91
0.0	0.9792	12.53
0.1	0.9802	11.85
0.1	0.9799	12.05
0.2	0.9809	11.37
0.2	0.9805	11.64
0.3	0.9806	11.57
0.3	0.9798	12.12
0.35	0.9803	11.78
0.35	0.9811	11.25
0.4	0.9807	11.51
0.4	0.9821	10.59
0.5	0.9810	11.31
0.5	0.9809	11.37
0.7	0.9805	11.64

An observation of Table 13 indicates no decided optimum for ammonium chloride. Some ammonium salts are present in the yeast

extract. The fermentative activity was greater, however, where an appreciable amount of ammonium chloride was present. A concentration of 0.15 grams per 100 ml. of medium was used in subsequent media.

The next nutrient studied was the phosphate. Potassium acid phosphates were used wherein half of the salt was the primary and the other half was the secondary salt. Results are shown in Table 14.

TABLE 14

EFFECT OF POTASSIUM ACID PHOSPHATE CONCENTRATION ON ETHANOL YIELD

Flasks: 200 ml. in 500 ml. Erlenmeyer flasks.
Medium: 15% dextrose, 0.375% yeast extract, 0.15% NH_4Cl, and other materials as in Table 11, except for the phosphate where the weight in each flask was made up of 50% primary and 50% secondary potassium phosphates, as designated below. pH = 5.
Sterilization: 13 lbs. for 20 minutes.
Inoculum: 10% dextrose medium, 5% inoculation.
Incubation: 4 days at 30° C.

Grams of phosphates in 200 ml.	Density of distillate, d_{25}^{25}	Grams of ethanol, Total
0.0	0.9797	12.19
0.0	0.9794	12.39
0.1	0.9795	12.32
0.1	0.9795	12.32
0.2	0.9795	12.32
0.2	0.9794	12.39
0.4	0.9794	12.39
0.4	0.9795	12.32
0.6	0.9799	12.05
0.6	0.9801	11.91

These salts do not seem to be critical in requirement. Some phosphates are no doubt present in the yeast extract allowing fermentation with no other phosphate added. Observation of the data and reaction vigor indicated that about 0.15 gram per 100 ml. of the mixed phosphates is advantageous.

The effect of varying concentrations of magnesium sulfate ($MgSO_4 \cdot 7H_2O$) was studied next. It was noted by Cennstein and Lüdecke

TABLE 15

EFFECT OF MAGNESIUM SULFATE CONCENTRATION ON ETHANOL YIELD

Flasks: 200 ml. in 500 ml. Erlenmeyer flasks.
Medium: 15% dextrose, 0.375% yeast extract, 0.15% NH_4Cl, 0.15% phosphate mixture, and other materials as in Table 11 except for $MgSO_4 \cdot 7H_2O$ which is as shown below. pH = 5.
Sterilization: 13 lbs. for 20 minutes.
Inoculum: 10% dextrose medium, 5% inoculation.
Incubation: 4 days at 30° C.

Grams of $MgSO_4 \cdot 7H_2O$ in 200 ml.	Density of distillate, d_{25}^{25}	Grams of ethanol Total
0	0.97930	12.46
0	0.97921	12.52
0.02	0.97930	12.46
0.02	0.97920	12.53
0.04	0.97920	12.53
0.04	0.97930	12.46
0.06	0.97930	12.46
0.06	0.97922	12.51
0.08	0.97925	12.50
0.08	0.97919	12.54

(1924) in their glycerol fermentation work that magnesium sulfate was important as a fermentation accelerator. Work was done by other investgators, but as their purpose was not to obtain a medium with minimum added solids, the investigation described in Table 15 was made.

The results of Table 15 obviously are inconclusive insofar as

TABLE 16

EFFECT OF CALCIUM CHLORIDE CONCENTRATION ON ETHANOL YIELD

Flasks: 200 ml. in 500 ml. Erlenmeyer flasks.
Medium: 15% dextrose, 0.375% yeast extract, 0.15% NH_4Cl, 0.15% phosphate mixture, 0.02% $MgSO_4 \cdot 7H_2O$ and other materials as in Table 11 except for $CaCl_2$ which is shown below. pH = 5.
Sterilization: 13 lbs. for 20 minutes.
Inoculum: 10% dextrose medium, 5% inoculation.
Incubation: 4 days at 30° C.

Grams of $CaCl_2$ in 200 ml.	Density of distillate d_{25}^{25}	Grams of ethanol total
0	0.97825	12.80
0	0.97800	12.97
0.02	0.97770	13.18
0.02	0.97745	13.35
0.04	0.97770	13.18
0.04	0.97780	13.11
0.06	0.97820	12.83
0.06	0.97850	12.63
0.08	0.97905	12.33
0.08	0.97780	13.11

optimum concentration of magnesium sulfate is concerned. However,

observations on the rate of fermentation indicated that the presence of some of the salt is to be desired. The flasks of higher magnesium sulfate concentrations were quiescent sooner than were the flasks of lower concentration. A concentration of .02 grams per 100 ml. of medium was used for subsequent media.

The effect of the concentration of calcium chloride on the ethanol yield from a yeast fermentation of dextrose was studied next. Data are found in Table 16. From the results of Table 16 it was decided to use a concentration of 0.01 grams of calcium chloride per 100 ml. of basal medium.

No particular advantage was found in the addition of an iron salt, so no iron salts were included in the final basal medium.

TABLE 17

OPTIMUM SEMI-SYNTHETIC MEDIUM FOR MAXIMUM ETHANOL PRODUCTION UTILIZING A MINIMUM OF ADDED SOLIDS

Reagent	Weight per 100 ml. of Medium
Yeast Extract (Difco anh.)	0.375 gm.
NH_4Cl	0.15 gm.
$K_2HPO_4 \cdot 3H_2O$	0.075 gm.
KH_2PO_4	0.075 gm.
$MgSO_4 \cdot 7H_2O$	0.02 gm.
$CaCl_2$	0.01 gm.
Dextrose for carrying culture	5.0 gm.
Dextrose for inoculum	10.0 gm.
Dextrose for experimental media	15.0 gm.

The optimum medium was taken to be as described in Table 17. The sugar content varied according to the purpose of the medium.

Tap water was used in making up the media.

The quantities of the salts employed in the medium were not found to be critical in the experimental series; hence, the basal medium described in Table 17 was chosen for the sake of consistency in the work.

3. The alkaline dissimilation of dextrose for the preparation of glycerol.

a. The use of ammonium hydroxide. This reagent was used by Krug and McDermott (1935) in their patent on the fermentation of sugar to glycerol. They obtained 15.8 to 18.5 percent glycerol based on percent of sugar weight, or 31 to 36.3 percent of the theoretical yield. Three liters of a molasses medium required a total of about 50 ml. of ammonium hydroxide (28 percent NH_3) which was added in portions. The dilution effect was not very great with that small an added volume. The ammonia was added at intervals to keep the pH near neutral. Usually the pH was about 7.2 or 7.3 immediately after the ammonia addition, but it soon approached neutrality or slight acidity (pH = 6.8). More ammonia was then added. In using ammonia, no "fixed" salts were added, and the glycerol was more easily recovered.

Some controlled fermentations were desired at various pH levels. It would be advantageous from a theoretical point of view to be able to express glycerol yield as a function of pH. The Cameron pH Recorder-Controller is to control fermentations at a desired pH level by automatically adding the base as required. The equipment was

prepared for the automatic addition of liquids so the addition of ammonium hydroxide solutions was possible.

Three liters of optimum medium were prepared in the usual way in a 5-liter, 3-necked flask. The experiment was known as trial "L". Ammonium hydroxide solution (14 percent NH_3) was prepared and placed in the reservoir for automatic addition. Inoculation was made with 300 ml. of yeast culture No. 43 and control was attempted at pH = 5 to try out the machine so that any difficulties arising in the process would be due to something other than the culture growth itself. Operation was fairly good for the first three days, but at the end of that time the medium became slightly less acid without the addition of more base, and as soon as the pH reached 5.1, control was lost and the whole of the ammonium hydroxide solution emptied into the medium making the pH = 9. The control had not yet been adapted for the condition in which the pH increased, rather than decreased from the value set by the instrument. A safety circuit was devised and has been described in the section above. The fermentation control was thus unsuccessful in this experiment except that it led to development of the safety circuit.

A second batch of medium was prepared as before. It was sterilized for 20 minutes. This was known as trial "M". The pH was controlled at 7.5 by ammonium hydroxide additions. The method was the same as with trial "L" except for the pH. The safety circuit was used to protect against losing pH control by a pH increase. The medium was

automatically stirred for 30 seconds of every four minutes, and also when the base was automatically added. The pH was four after inoculation, but it was brought to about 7.5 gradually over a period of five hours. The machine worked excellently, but the fermentation was found to have ceased after about one day at this pH. Sugar was not being consumed, and no base was added.

Among the reasons considered for the death of the culture were:

(1) Lack of acclimatization of the culture to the abnormal conditions.

(2) Toxicity related in some manner to the ammonium ion.

(3) Unfavorable pH.

(4) Too rapid a pH change in the beginning.

(5) Culture too weak at the start of the control period.

A batch of medium was prepared as above which was to be controlled at pH = 6.8 by ammonium hydroxide (14 percent NH_3). This was known as trial "N". The pH was controlled at five for 12 hours following inoculation. Vigorous growth resulted. The pH was gradually brought to 6.8, the process requiring a period of about nine hours. After the culture was 90 hours old, control was found to be unnecessary and was shut off while allowing the recording to proceed. The pH had risen to about 7.3 after 118 hours. This seems to be a typical phenomenon. At 96 hours and at 118 hours about 0.5 percent of the original sugar was found remaining. The pH was checked at intervals with a Cameron pH Meter; the record was satisfactory. Analyses showed 13.8 grams of glycerol and 36.6 grams of ethanol were obtained from 100 grams of

dextrose, or 27.1 percent of the theoretical glycerol, or 13.8 percent on the sugar basis. About 99.5 percent of the sugar was consumed. About 92 percent of the dextrose was accounted for, taking into consideration the fact that some ethanol is formed by the third equation of Neuberg, and the rest by the first equation (alcoholic). The glycerol yield of this fermentation might be expected to be a bit lower than those reported by Krug and McDermott (1935) as this was run at pH 6.8 to give 13.8 percent glycerol on the sugar, while they varied the pH from 6.8 to about 7.3 and obtained 15.8 to 18.5 percent of the sugar as glycerol. Another difference was that they used molasses instead of dextrose.

The next trial, known as "O", was run as before, except that some solid calcium carbonate was added to the medium to keep the pH nearer neutral before the control was applied. About 10 grams of calcium carbonate were added to the three liters of medium. After inoculation, the culture grew luxuriantly for 24 hours before control was applied. Some instrumental difficulties had arisen. The pH of the medium at 24 hours with calcium carbonate present was about 5.8. Ammonia water was added as usual for about six hours to bring the pH to 7.7 for the completion of the fermentation. With such a strong start the fermentation should have gone well. However, fermentation ceased with 33 percent of the sugar untouched.

The next trial, known as "P", was run in the usual way. The pH was gradually brought to 7.5 very carefully over a period of nine

hours after inoculation. But this run was inhibited as were the rest of the unsuccessful experiments at high pH values.

With such consistent unsuccessful fermentations at controlled pH values over pH = 7, it was thought that perhaps the difficulty could be associated with the use of ammonium hydroxide rather than pH itself. Also, Krug and McDermott (1935) did not describe fermentations with average pH much more than seven which were successful, although they claimed the pH range from seven to eight to be desirable. There have been numerous references to alkaline fermentations in the literature, so the pH alone is not the cause of the lack of completion of the fermentations described above. The controlled fermentations "I" through "P" are compared in Table 18. They are arranged in the order of the controlled pH values.

b. Studies with ammonium salt buffers. Because of the results of the above experiments, and because of the fact that only one experiment can be run at a time on the instrument, it was decided to study the effects of other ammonium salts on the fermentation in order to obtain systems of ammonium salt buffers which could be used to study the fermentation at various pH levels in numerous flasks simultaneously. An acid reacting ammonium salt such as ammonium chloride might be used with an alkaline reacting salt such as ammonium carbonate in various proportions to make a buffer series. The ammonium carbonate would tend to hydrolyze somewhat forming some ammonium hydroxide.

The presence of NH_4Cl would tend to suppress the dissociation of the ammonium hydroxide thus making the pH in effect more acid.

TABLE 18

FERMENTATIONS HAVING pH AUTOMATICALLY CONTROLLED BY AMMONIUM HYDROXIDE

Flask: 5 liter, 3-necked, containing 3 liters of medium.
Medium: Optimum semi-synthetic with 15% dextrose; 3 liters; pH = 5 at start.
Sterilization: 30 minutes at 10 lbs. steam pressure.
Inoculum: 300 ml. of culture No. 43 in 10% dextrose medium.
Incubation: 4 to 6 days at 30 to 32° C.
Instrument: Cameron pH Controller-Recorder.
Basic reagent: Ammonium hydroxide (14 percent NH_3)
Stirring: Automatic, 30 sec. out of 4 min., and on addition of base.

Trial	pH	Ethanol Yield, % on sugar	Glycerol Yield, % on sugar	Residual Sugar, % unferm.	Remarks
L	5.0	-	-	-	Mechanical difficulties. Control lost. Excess NH_4OH added automatically.
N	6.8	36.6%	13.8%	0.48%	Successful.
M	7.4 to 7.5	-	-	Over 50	Mechanical operation excellent. Dissimilation ceased.
P	7.5	-	-	Over 50	Mechanical operation excellent, but growth ceased.
O	7.7	-	-	33	Solid $CaCO_3$ present. Mechanical operation bad the 1st day, so that medium had a 24 hour start of growth. But after pH was controlled at 7.7, growth ceased.

TABLE 19

THE EFFECT OF AMMONIUM CHLORIDE IN GREATER THAN NUTRIENT QUANTITIES ON THE ALCOHOLIC FERMENTATION OF THE OPTIMUM SEMI-SYNTHETIC MEDIUM

Medium: Optimum, with added salt as shown. 300 ml. in 500 ml. Erlenmeyer flasks; Contained 15% dextrose; Initial pH = 5.
Inoculum: 20 ml. of Culture No. 43 in 10% dextrose optimum medium.
Incubation: 7 days at 30° C. (Long time because of incompletely fermented media with high salt concentration.)

Flask No.	Total NH_4Cl, gm. per 100 ml.	NH_4Cl Normality.	Final pH	Ethanol Yield		Glycerol Yield			Residual Sugar, % Unferm.
				Gms., corrected for Inoculum	% of Theory	Gm. per 100 ml. Medium	% of Theory	% on Sugar	
1	0.15	0.0282	2.96	17.44	75.8%	0.642	8.01%	4.09%	0.47%
2	0.25	0.0469	2.96	17.37	75.5	0.640	7.99	4.07	0.39
3	0.40	0.0750	2.97	17.97	78.1	0.676	8.45	4.31	0.35
4	0.60	0.1124	2.95	18.04	78.5	0.688	8.59	4.38	0.44
5	0.80	0.149	2.92	18.11	78.8	0.702	8.75	4.46	0.38
6	1.00	0.179	2.92	18.11	78.8	0.720	8.99	4.58	0.41
7	1.50	0.280	2.98	17.71	77.0	0.746	9.31	4.75	1.07
8	2.00	0.374	3.01	17.71	77.0	0.748	9.35	4.76	2.17
9	3.00	0.561	3.01	13.15	57.2	0.735	9.17	4.67	16.0
10	4.00	0.748	3.10	9.90	43.1	-	-	-	39.4
11	5.00	0.935	3.22	5.01	21.8	-	-	-	65.2

It was first desired to ascertain what maximum concentration of ammonium chloride could be used in a buffer without suppressing or inhibiting the fermentation. An experiment was designed to show the effect of ammonium chloride on fermentations of media which are optimum except for the ammonium chloride concentration. The results and data are shown in Table 19.

Observation of Table 19 shows that the sugar was consumed satisfactorily when the ammonium chloride concentration was below 1.5 grams per 100 ml. At higher concentrations of the salt, the unfermented sugar content rose rapidly. There seemed to be a gradual increase in the glycerol formed as the salt content increased. The data on resulting glycerol are given also in Table 19. It was suspected at this point that perhaps the chloride ion might have been the inhibiting factor, rather than both the ammonium and chloride ions. A series of fermentations was designed to study the effect of ammonium carbonate on the fermentations by adding varying amounts to the medium. The results are given in Table 20.

It may be noted again that when the pH was appreciably greater than 7, no growth occurred. The yield of glycerol increased as the final pH increased up to the point where growth did not occur. Flask "O", having the same ammonium normality as flask "7" of Table 19 but less chloride, had less residual sugar than flask "7". This was to have been expected if chloride ions were the inhibitors.

Although it was considered that the chloride ion concentration

TABLE 20

THE EFFECT OF AMMONIUM CARBONATE ON PRODUCTS, EXTENT, AND pH OF FERMENTATIONS WITH YEAST

Medium: Optimum, with salt added as shown. 300 ml. in 500 ml. Erlenmeyer flasks; Contained 15% dextrose; Initial pH as shown, approximately; HCl was added to all but A in small amounts as the media seemed too alkaline.

Inoculum: 22 ml. of Culture No. 43 in 10% dextrose optimum medium added to all flasks but E, F, and G which received 12 ml.

Incubation: 6 days at 30° C.

Flask No.	$(NH_4)_2CO_3$ added, gm. 100 ml.	NH_4^+ Normality. Total	Initial pH (approximate)	Final pH	Ethanol yield: Gm. corr. for Inoc.	Ethanol yield: % of Theory	Glycerol yield: Gm. per 100 ml. of Medium	Glycerol yield: % of Theory	Glycerol yield: % on Sugar	Residual Sugar, % Unferm.
A	0.000	0.0282	5	2.86	19.30	84.0%	0.610	8.11%	4.14%	0.26%
B	0.0898	0.0469	6	3.59	19.24	83.7	0.665	8.85	4.51	0.26
C	0.224	0.0750	6	3.94	18.72	81.5	0.815	10.83	5.53	0.23
D	0.404	0.1124	6	4.32	18.10	78.8	0.855	11.38	5.80	0.23
E	0.580	0.149	6	4.40	17.92	78.0	1.065	14.17	7.23	0.23
F	0.763	0.179	6	5.00	17.78	77.4	1.1025	14.6	7.48	0.24
G	1.210	0.280	6	6.37	17.92	78.0	1.2525	16.66	8.50	0.26
H	1.66	0.374	8	7+	fermentation ceased			-	-	high
I	2.56	0.561	8	7+	fermentation ceased			-	-	high
J	3.46	0.748	8	7+	fermentation ceased			-	-	high
K	4.36	0.935	8	7+	fermentation ceased			-	-	high

was the inhibiting factor in fermentations of acid pH and of relatively high ammonium chloride concentrations, it was desired to know the type and the extent of effectiveness of various mixtures of ammonium chloride and ammonium carbonate as buffers for possible use in fermentations. The ammonium carbonate used in all cases was the monohydrate but weights were calculated on the anhydrous basis. Normal

TABLE 21

EFFECT OF AMMONIUM CHLORIDE-AMMONIUM CARBONATE RATIO ON THE pH OF SOLUTIONS OF CONSTANT AMMONIUM ION NORMALITY (Temperature = 25° C.)

Equivalent % of $(NH_4)_2CO_3$	Equivalent % of NH_4Cl	pH of Solutions 1 N. in NH_4^+.	pH of Solutions 0.5 N. in NH_4^+.
0%	100%	4.89	5.03
1	99	6.94	7.09
2	98	7.15	7.30
4	96	7.40	7.52
10	90	7.74	7.91
20	80	8.00	8.20
40	60	8.27	8.48
60	40	8.43	8.67
80	20	8.58	8.79
100	0	8.72	8.92

solutions of both ammonium chloride and ammonium carbonate were prepared in distilled water. Various mixtures of the two solutions were made up which resulted in a constant ammonium ion concentration, but with the anions of varying equivalent proportions. The pH of these solutions was measured at 25° centigrade by means of the Cameron

glass electrode pH meter. After these measurements were made, the solutions were diluted with an equal volume of distilled water. The resulting solutions were 0.5 normal in ammonium ions. The pH measurements were again made as before. The data are given in Table 21 and Figure 5.

The data in Table 21 indicate that such mixtures of ammonium salts might be satisfactory for buffered pH fermentations if other factors do not make their use impractical. Other ammonium salts such as the sulfate should act in a manner similar to the action of ammonium chloride in such buffers. A fairly high concentration of ammonium carbonate should be used so that the acetic acid that forms during the fermentation would convert only a small fraction of the carbonate to acetate.

In order to have some data regarding the inhibiting effects of various anions of ammonium salts on alcoholic fermentations of the optimum medium being studied, where the salt concentration is greater than nutrient requirements, the fermentations described in Table 22 and in Figure 6 show what salt or salts may be used in concentrations approaching 1 normal. It was shown in Table 19 that ammonium chloride could not be used in concentrations above about 0.25 normal without leaving more than 0.5 percent of the sugar unfermented. The chloride, sulfate, and nitrate were studied separately and as mixtures.

The results proved that ammonium sulfate was by far the most

TABLE 22

THE EFFECT OF SEVERAL AMMONIUM SALTS IN CONCENTRATIONS APPROACHING 1 NORMAL ON THE ALCOHOLIC FERMENTATION

Medium: Optimum, but with salts added as shown; 300 ml. in 500 ml. Erlenmeyer flasks; 15% dextrose; Initial pH = 5.
Inoculum: 20 ml. of Culture No. 43 in optimum medium of 10% dextrose.
Incubation: 6 days at 30° C.

Flask No.	NH_4^+ Normality	Total Gm. of NH_4Cl Added	Total Gm. of NH_4NO_3 Added	Total Gm. of $(NH_4)_2SO_4$ Added	Final pH	Dextrose Remaining: Gm. per 100 ml. Medium	Dextrose Remaining: Percent Unfermented
A8	0.374	5.55	0	0	2.9	5.22	35.5%
A9	0.561	8.55	0	0	2.9	5.15	35.1
A10	0.748	11.55	0	0	2.9 +	11.10	75.5
A11	0.935	14.55	0	0	3.0	11.78	80.3
B8	0.374	0	8.30	0	2.9	3.38	23.0
B9	0.561	0	12.8	0	2.9	4.55	31.0
B10	0.748	0	17.3	0	2.9 +	5.90	40.2
B11	0.935	0	21.8	0	3.0	7.85	53.5
C8	0.374	0	0	6.85	2.9	0.068	0.46
C9	0.561	0	0	10.5	2.9	0.036	0.25
C10	0.748	0	0	14.2	2.9 +	0.18	1.225
C11	0.935	0	0	17.9	3.0	0.96	6.54

TABLE 22 (continued)

THE EFFECT OF SEVERAL AMMONIUM SALTS IN CONCENTRATIONS APPROACHING 1 NORMAL ON THE ALCOHOLIC FERMENTATION

Medium: Optimum, but with salts added as shown; 300 ml. in 500 ml. Erlenmeyer flasks; 15% dextrose; Initial pH = 5.
Inoculum: 20 ml. of Culture No. 43 in optimum medium of 10% dextrose.
Incubation: 6 days at 30° C.

Flask No.	NH_4^+ Normality	Total Gm. of NH_4Cl Added	Total Gm. of NH_4NO_3 Added	Total Gm. of $(NH_4)_2SO_4$ Added	Final pH	Dextrose Remaining: Gm. per 100 ml. Medium	Dextrose Remaining: Percent Unfermented
D8	0.374	2.78	4.15	0	2.9	1.96	13.34
D9	0.561	4.28	6.4	0	2.9	4.70	32.0
D10	0.748	6.78	8.65	0	2.9 +	7.60	51.75
D11	0.935	8.28	10.9	0	3.0	9.19	62.50
E8	0.374	0	4.15	3.43	2.9	0.94	0.64
E9	0.561	0	6.4	5.3	2.9	1.596	10.87
E10	0.748	0	8.65	7.1	2.9 +	3.65	24.90
E11	0.935	0	10.9	9.0	3.0	3.86	26.30
F8	0.374	1.85	2.8	2.28	2.9	0.60	4.09
F9	0.561	2.85	4.3	3.5	2.9	1.588	10.80
F10	0.748	3.85	5.8	4.7	2.9 +	4.38	29.82
F11	0.935	4.85	7.3	6.0	3.0	5.96	40.60
Control	0.0282	0	0	0	2.9	0.068	0.46

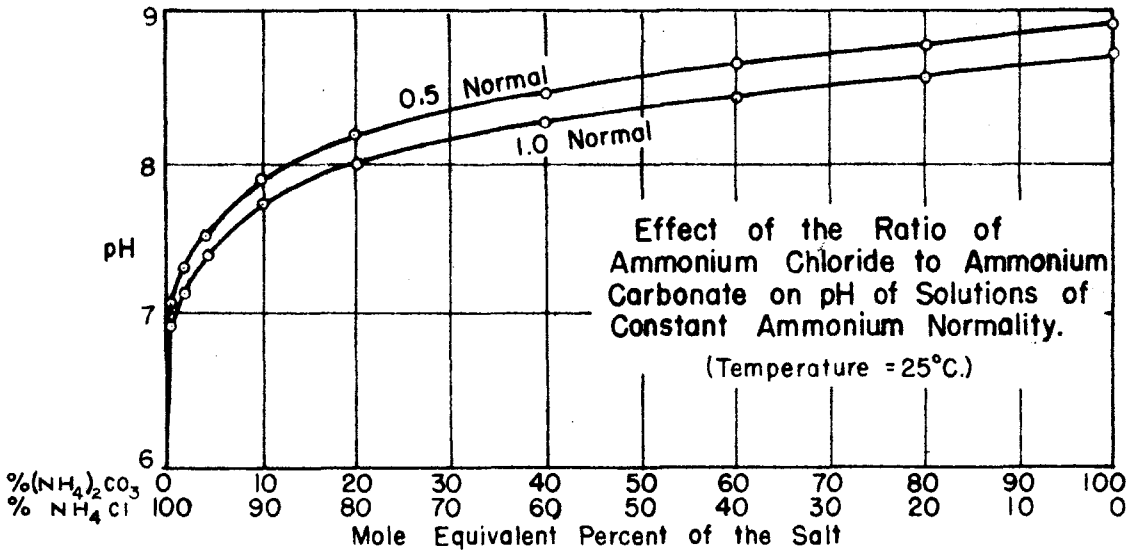


Figure 5

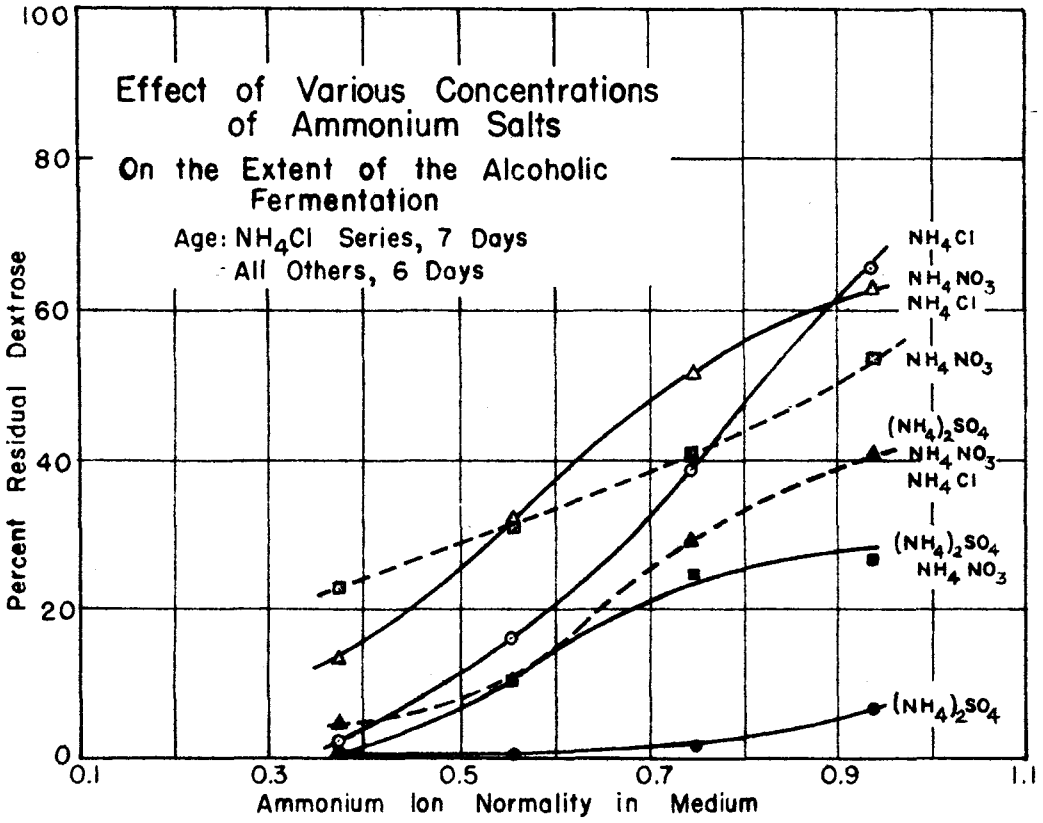


Figure 6

satisfactory salt of those studied, insofar as residual sugar was concerned. It was also shown that the ammonium ion was not the inhibiting factor as much as was the chloride ion. Both chloride and nitrate were unsatisfactory in concentrations up to about 0.5 normal, while the sulfate was satisfactory. Mixtures of the various salts were not as satisfactory as was ammonium sulfate alone. It is well to note that all these fermentations were of the straight alcoholic type, i.e., the pH was about three and sulfite was absent. Buffer mixtures of ammonium carbonate and ammonium sulfate should be satisfactory for studying the pH effect on glycerol formation during sugar breakdowns by yeast, with the ammonium ion present. It should be remembered that ammonium salts are desirable rather than sodium or potassium salts because the cation may be removed by volatilization after alkalization.

A complete series of fermentations was desired where pH is controlled by ammonium salt buffers where the trend of a number of fermentations might be studied at one time. Such studies would take much too long a time with the automatic controller which can control but one fermentation at a time. Since it was shown in Table 22 that ammonium sulfate could be used satisfactorily in concentrations somewhat greater than 0.5 normal, it was decided to prepare a series of fermentations containing various mixtures of ammonium sulfate and ammonium carbonate along with the small amount of ammonium chloride added as a nutrient to the optimum medium. The

total ammonium ion concentration was made to be 0.5 normal in all cases. The experiment is described in Table 23; the results and implications may be more easily observed in Figure 7.

Once again it is obvious that the fermentations went to completion quite well as long as the fermenting medium had a pH below 7, approximately. As soon as the pH exceeded 7, the rate of sugar consumption fell off quite rapidly and the growth was sluggish. Although the media were not buffered at exactly fixed pH values, the values for most of the media were near enough to a fixed value so that trends could be noted and conclusions may be drawn within reasonable limits of error.

The technique used was not for the purpose of getting maximum possible glycerol yields, but rather to study the fermentative trends. The glycerol yield increased with the pH as the alcohol decreased, up to approximately pH = 7. The more alkaline media contained higher residual sugar than the media having pH values below 7.

Evidently something happened at about pH = 7, when ammonium salts were used in relatively large quantities, which caused inhibition of dissimilation. This effect was not shown in the alkaline fermentations using soda ash. The change that seemed obvious as the pH approached and then passed 7 was the very rapid formation of molecular ammonium hydroxide which is in equilibrium with molecular ammonia. The entire set of equilibria is as follows:

$$NH_4^+ + OH^- \rightleftharpoons NH_4OH \rightleftharpoons H_2O + NH_3(sol'n) \rightleftharpoons NH_3(gas)$$

TABLE 23

AMMONIUM SULFATE-CARBONATE BUFFERS 0.5 NORMAL IN AMMONIUM ION CONCENTRATION; THEIR EFFECT ON pH, GLYCEROL FORMED, AND ON SUGAR CONSUMPTION

Medium: Optimum with 15% dextrose. Salts were added as indicated. Both salts were added to concentrated media as 3 N. solutions. All the sulfate was added at the start, but the carbonate was added in portions over periods of time proportional to the amount to be added (after inoculation). All the carbonate was added by the end of about 24 hours in flasks Q, R, S, and T. The final medium in each 500 ml. flask was 320 ml. of optimum medium except for the added salts of the buffer. The ammonium concentration was 0.5 N. when the optimum NH_4Cl already there is considered.

Inoculum: 20 ml. of 10% dextrose culture of yeast No. 43.

Incubation: 6 days at 30° C.

Flask	Vol. of $(NH_4)_2CO_3$, ml. 3 N.	Vol. of $(NH_4)_2SO_4$, ml. 3 N.	pH 20 hr.	pH 48 hr.	pH 68 hr.	pH 96 hr.	pH 112 hr.	pH 145 hr.	Glycerol found: % of Theory	Glycerol found: % on Sugar	Ethanol Found % of Theory	Dextrose, % Unferm.
A	0	50	3.50	-	3.41	3.34	3.39	3.29	11.3%	5.75%	80.0%	0.71%
B	1/4	49 3/4	3.58	-	3.40	3.34	3.27	3.31	10.3	5.25	80.3	0.64
C	1/2	49 1/2	3.70	-	3.43	3.35	3.32	3.33	11.0	5.60	80.1	0.75
D	3/4	49 1/4	3.88	-	3.54	3.42	3.43	3.42	10.9	5.55	81.8	0.61
G	2	48	4.78	3.73	3.66	3.70	3.75	3.72	11.05	5.64	80.1	0.53
I	4	46	5.34	4.28	4.12	4.17	4.17	4.14	12.53	6.40	79.0	0.61
L	8	42	5.83	5.43	5.20	5.21	5.18	5.29	15.52	7.92	74.0	0.53
M	12	38	6.15	6.09	5.95	6.02	6.19	6.41	15.42	7.86	71.3	0.32
N	18	32	6.39	6.42	6.39	6.49	6.54	6.80	16.77	8.55	69.0	0.32
O	25	24	6.41	6.57	6.51	6.66	6.73	6.86	19.1	9.74	67.2	1.42
P	30	20	6.49	6.78	6.78	6.82	6.87	7.03	20.0	10.2	60.9	7.25

TABLE 23 (continued)

AMMONIUM SULFATE-CARBONATE BUFFERS 0.5 NORMAL IN AMMONIUM ION CONCENTRATION; THEIR EFFECT ON pH, GLYCEROL FORMED, AND ON SUGAR CONSUMPTION

Medium: Optimum with 15% dextrose. Salts were added as indicated. Both salts were added to concentrated media as 3 N. solutions. All the sulfate was added at the start, but the carbonate was added in portions over periods of time proportional to the amount to be added (after inoculation). All the carbonate was added by the end of about 24 hours in flasks Q, R, S, and T. The final medium in each 500 ml. flask was 320 ml. of optimum medium except for the added salts of the buffer. The ammonium concentration was 0.5 N. when the optimum NH_4Cl already there is considered.

Inoculum: 20 ml. of 10% dextrose culture of yeast No. 43.

Incubation: 6 days at 30° C.

Flask	Vol. of $(NH_4)_2CO_3$, ml. 3 N.	Vol. of $(NH_4)_2SO_4$, ml. 3 N.	pH 20 hr.	pH 48 hr.	pH 68 hr.	pH 96 hr.	pH 112 hr.	pH 145 hr.	Glycerol found: % of Theory	Glycerol found: % on Sugar	Ethanol Found % of Theory	Dextrose, % Unferm.
Q	35	15	6.61	6.94	6.88	6.90	7.01	7.19	16.0	8.15	41.8	33.8
R	40	10	6.67	7.05	7.02	7.21	7.48	7.71	6.03	3.08	24.2	60.6
S*	45	5	7.17	7.12	7.18	7.41	7.63	7.80	8.06	4.11	26.8	50.2
T*	50	0	7.18	7.27	7.34	7.60	7.79	7.91	2.45	1.25	28.3	56.2
X	0	0	3.50	2.95	2.95	3.01	3.05	3.06	10.6	5.40	-	0.5

Flasks marked (*) were each inoculated with 20 ml. from flask L at the age of 48 hours.
Flask X is a control with no buffering salts added.

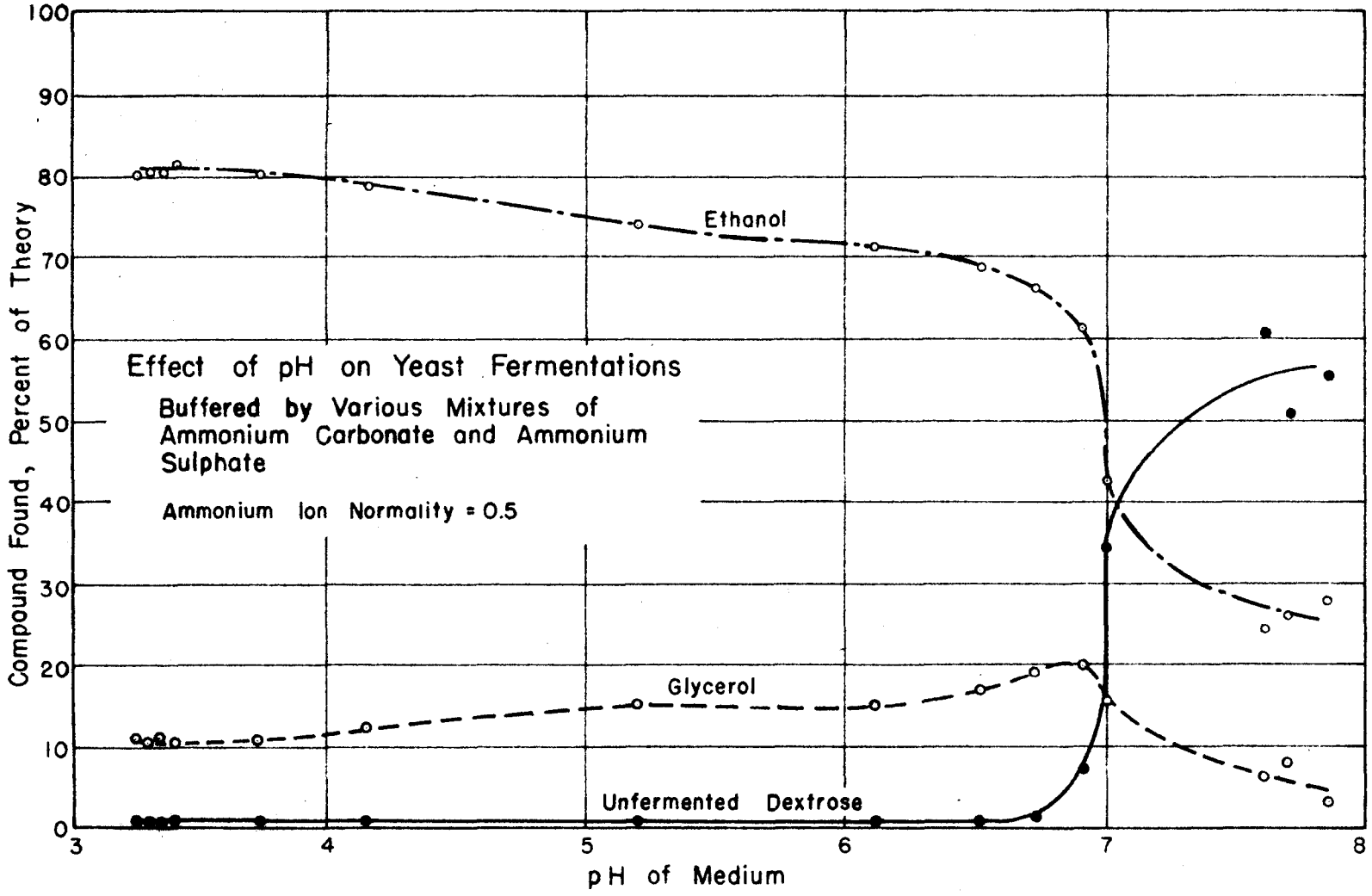

Effect of pH on Yeast Fermentations
Buffered by Various Mixtures of Ammonium Carbonate and Ammonium Sulphate
Ammonium Ion Normality = 0.5
Ethanol
Glycerol
Unfermented Dextrose
Compound Found, Percent of Theory
pH of Medium
100
90
80
70
60
50
40
30
20
10
0
3
4
5
6
7
8

Figure 7

The ammonium ion was not at fault, nor was the sulfate, as was shown in Table 22. Carbonate ions were not at fault, as soda ash fermentations were successful. Ammonia gas was, of course, out of the medium itself. The compounds left for suspicion were the molecular ammonium hydroxide, and molecular ammonia in solution. The concentrations of these agents were probably not as important as were the chemical activities as defined by Lewis and Randall (1923), or the chemical potentials, as they are also known. The relation of chemical potential to bacteriology has been discussed by Buchanan and Fulmer (1930a).

The activity of molecular ammonia, for example, is practically negligible at pH values more acid than about pH = 6.8, while the activity begins to appear and rise very rapidly as the pH passes 7 and goes on to 8 or more. As pH = 7 is reached and passed, the hydroxide ion concentration increases very rapidly. This allows the formation of more and more ammonium hydroxide molecules which, being in equilibrium with ammonia, increases the activity of molecular ammonia. The presence of appreciable concentrations of ammonium salts at a given pH will have the effect of increasing the activity of the ammonia over that activity or chemical potential in solutions of low ammonium salt concentrations. This might be considered in some respects analogous to the increase in bactericidal activity of phenol solutions brought about by added sodium chloride. The added salt decreases the solubility of the phenol, thus bringing the resulting solution nearer to saturation. The phenol then has a

greater "escaping tendency". Such an effect signifies greater chemical activity or potential. The phenol is, in effect, driven more forcibly into the organism. Likewise, ammonia would be less soluble in a solution of high ammonium salt concentration than in one of low concentration, and the ammonia activity would thus be greater. A fixed concentration of ammonia would have a greater activity in a 2 normal solution of sodium carbonate than it would have in a 1 normal solution of the same salt - other factors remaining the same. The activity would also be increased with an increase in pH.

This theory explains every successful and every unsuccessful fermentation mentioned thus far where ammonium concentrations over nutrient quantities were used. Removal of most of the ammonium salts should allow fermentations at greater alkalinity than those where ammonium salts are present. It will be noted later that the first fermentation controlled by soda ash was successful at pH 7.5.

Krug and McDermott (1935) obtained glycerol yields from the fermentation of molasses media which were 15.8 to 18.5 percent of the sugar weight, whereas the maximum obtained as shown in Table 23 was about 10 percent of the total sugar weight, or about 11 to 12.3 percent on the sugar fermented in flasks "P" and "Q". However, the available ammonium ion concentration in the experiments of Krug and McDermott was about 0.26 to 0.28 normal, while the experiments of Table 23 were run in media which were 0.5 normal in available ammonium

ion concentration. Near pH = 7 much of the ammonium ions would be converted to molecular ammonium hydroxide and ammonia, the activity, or chemical potential, of which would increase as a function of increasing pH and of increasing ammonium or ammonia concentrations. Thus the ammonia activity would be greater at a given pH in the experiments of Table 23 than in the fermentations of Krug and McDermott since more ammonium ions were available for ammonia formation near the neutral pH. Then a lower pH would be required for fermentations of high available ammonia in order to decrease the activity of the ammonia enough so that inhibition of growth is not predominant. Thus the higher yields would not be expected in Table 23. Also Krug and McDermott used about 16 to 18 grams of molasses sugars per 100 cc. of medium, while 15 grams per 100 ml. were employed in Table 23. The bio-colloids of the molasses may have had some effect also, according to Owen (1937). The results thus far are perhaps reasonable, though not identical. Higher sugar concentrations are conducive to greater glycerol formation according to McDermott (1929). He also noted that molasses media in the presence of relatively large amounts of sodium carbonate gave a slight precipitation along with the development of a slight ammoniacal odor. The ammonia activity in the molasses might limit the maximum pH at which fermentation would be complete. The odor of ammonia would be obvious evidence of its presence and activity.

An experiment, described below in the section dealing with the

ammonium sulfite fermentation, further showed the difficulties of using very high concentrations of ammonium salts in fermentations near pH = 7 or over.

It was desired to obtain evidence of a more quantitative nature concerning the toxic effects of free ammonia or ammonium hydroxide in fermentation media. Several series of fermentations were designed to show that at constant pH the toxicity increases as a function of the initial ammonium ion concentration. The series were also designed to show that at constant initial ammonium ion concentration the toxicity increases with increasing pH and that pH = 7 is the critical value.

A semi-synthetic medium was prepared which was optimum except that a nitrogen equivalent of urea was substituted for ammonium chloride in order to have the ammonium concentration equal to zero. It was prepared in 300-ml. Erlenmeyer flasks such that 180 ml. on dilution to 200 ml. would result in a 15 percent dextrose medium. Ammonium sulfate was added to each flask in 20 ml. of aqueous solution to give the media the desired ammonium ion concentrations. Sterilization was at 15 pounds for 15 minutes. The media of the various series were inoculated with 20 ml. of inoculum grown in 10 percent dextrose medium. The organism was S. cerevisiae No. 43. The cultures were incubated for 100 hours at 30° centigrade. The pH adjustments were made at frequent intervals by additions of solid sodium carbonate. Data are given in Table 24 and in Figure 8.

TABLE 24

SUGAR CONSUMPTION IN FERMENTATIONS AS RELATED TO pH AND AMMONIUM CONCENTRATION

$(NH_4)_2SO_4$ Normality	pH = 8.0		pH = 7.5	
	Grams of Na_2CO_3 Added per Flask	Residual Dextrose, gm./100ml.	Grams of Na_2CO_3 Added per Flask	Residual Dextrose, gm./100ml.
0.0	14.5 gm.	2.34 gm.	11.3 gm.	1.25 gm.
0.1	13.0	3.50	10.3	1.044
0.2	8.4	7.78	8.8	7.46
0.3	5.4	11.36	4.8	11.92
0.4	4.7	12.36	3.4	14.26
0.5	4.2	13.00	3.2	14.50

$(NH_4)_2SO_4$ Normality	pH = 7.2		pH = 7.0	
	Grams of Na_2CO_3 Added per Flask	Residual Dextrose, gm./100ml.	Grams of Na_2CO_3 Added per Flask	Residual Dextrose, gm./100ml.
0.0	5.6 gm.	0.50 gm.	3.5 gm.	0.74 gm.
0.1	4.7	0.05	3.4	0.16
0.2	4.5	0.76	3.4	0.41
0.3	4.4	5.44	3.3	0.82
0.4	4.1	9.24	3.3	3.34
0.5	3.8	10.70	3.2	8.22

$(NH_4)_2SO_4$ Normality	pH = 6.8		pH = 6.5	
	Grams of Na_2CO_3 Added per Flask	Residual Dextrose, gm./100ml.	Grams of Na_2CO_3 Added per Flask	Residual Dextrose, gm./100ml.
0.0	2.1 gm.	0.40 gm.	0.98 gm.	0.12 gm.
0.1	2.4	0.18	1.03	0.11
0.2	2.3	0.19	1.05	0.13
0.3	2.3	0.30	1.16	0.11
0.4	2.2	0.61	1.16	0.14
0.5	2.2	2.89	1.21	0.16

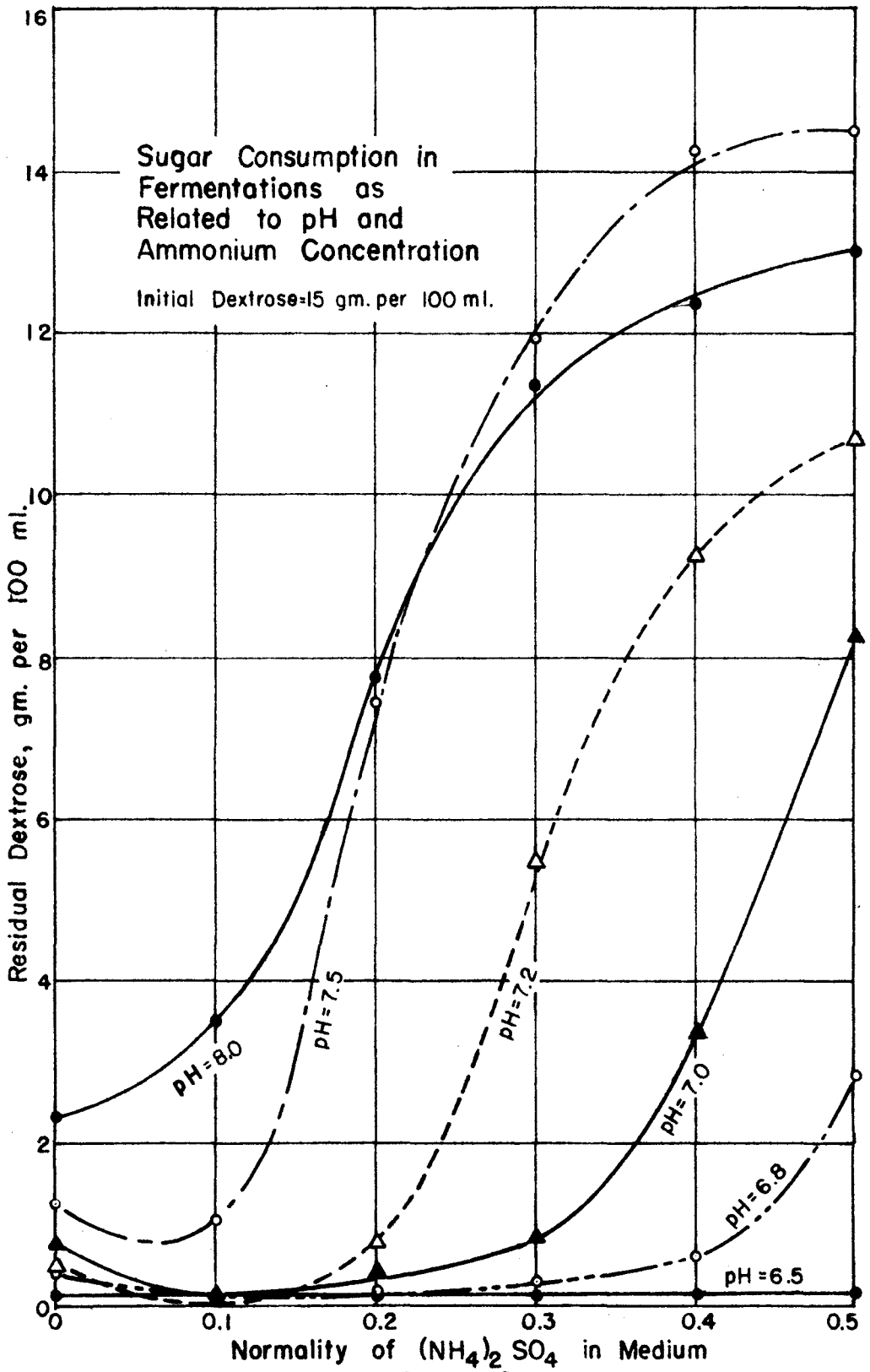

Sugar Consumption in Fermentations as Related to pH and Ammonium Concentration
Initial Dextrose=15 gm. per 100 ml.
Residual Dextrose, gm. per 100 ml.
Normality of $(NH_4)_2 SO_4$ in Medium
pH=8.0
pH=7.5
pH=7.2
pH=7.0
pH=6.8
pH=6.5
0
2
4
6
8
10
12
14
16
0
0.1
0.2
0.3
0.4
0.5

Figure 8

From the results indicated in Table 24 and in Figure 8, it is obvious that at constant pH near or above 7, increasing the ammonium concentration decreases dissimilation of the dextrose. One may also conclude that at constant ammonium concentration, increasing pH decreases the sugar dissimilation. This evidence substantiates the previous evidence that ammonia or ammonium hydroxide can exhibit toxic effects on yeast cultures. It is undoubtedly true that the toxicity is a function of the activity of the ammonia or ammonium hydroxide. The ammonium ion concentrations up to 0.5 normal were inhibitive in acid solution as shown by the fairly complete sugar consumption at pH = 6.5. Mathematical relationships could be derived, no doubt, between the toxicity and ammonia activity. However, since the main purpose of the toxicity investigations was attained, no further work in these lines was considered necessary.

It may be noted in Table 24 that the sugar was generally consumed more extensively when the ammonium ion concentration was 0.1 normal than when it was zero. It should be remembered that urea was substituted for ammonium chloride in the initial optimum medium. A discussion concerning the use of ammonia and urea in the nitrogen metabolism of yeast was given by Schultz, Atkin and Frey (1940).

Thus, since it was proven beyond doubt that the use of ammonium hydroxide and other ammonium agents for alkaline fermentations was impractical, attention was focused on other types of fermentations

for glycerol production.

c. The use of sodium carbonate. Sodium carbonate has been considered very extensively, after the work of Neff (1918) and of Neff, Linder and Beyer (1919), as an agent to bring about the formation of increased amounts of glycerol from the altered alcoholic fermentation. The sodium carbonate process has been generally impractical because of the recovery difficulties previously mentioned which were caused

TABLE 25

FERMENTATION AUTOMATICALLY CONTROLLED AT pH 7.5 BY SODIUM CARBONATE SOLUTION IN THE ABSENCE OF APPRECIABLE AMMONIUM SALTS

Flask:	5 liter, 3-necked, containing 3 liters of 15% medium at start.
Medium:	15% dextrose, optimum except that NH_4Cl was replaced by a nitrogen equivalent of urea, to remove ammonia effect. pH = 5 initially.
Sterilization:	30 minutes at 10 lbs.
Inoculum:	300 ml. of 10% dextrose culture. Yeast No. 43.
Incubation:	5 1/2 days at 32° C.
Aeration:	mild and intermittent for the first 7 hours.
Instrument:	Cameron pH Recorder-Controller.
Residual Dextrose:	0.35% of that originally present.
Glycerol found:	20% of theory, or 10.2% of the sugar weight.
Sodium carbonate:	added by solution (150 gm. Na_2CO_3 per liter of solution)

by the high salt concentrations necessary.

As was indicated under the ammonium hydroxide section, appreciable quantities of ammonium salts appear to cause inhibition or suppression of fermentation when the pH allows active ammonia to be present. A trial fermentation at an automatically controlled pH

value of 7.5 was desired, but no fermentation had been completed successfully with an appreciable ammonium ion concentration present at this degree of alkalinity.

Three liters of optimum medium were sterilized in a 5-liter, 3-necked flask. The experimental data are given in Table 25.

The fermentation partially described in Table 25 was automatically controlled at pH = 7.5 by a sodium carbonate solution containing 150 grams of the anhydrous salt per liter of solution. The fermentation was fairly rapid. The pH was controlled at 6.8 for about the first 20 hours, to allow a good growth of yeast to get under way; then the pH was changed to 7.5 over a period of two hours and held at that value. Fifty-one hours after inoculation, 1660 ml. of the alkaline solution had been added, corresponding to 249 grams of sodium carbonate. The total volume in the fermentation flask was rapidly approaching the 5 liter flask capacity by this time. Therefore, 500 ml. of the mixture was removed at this point. This procedure caused complications in the analytical computations. In 96 hours a total of 1945 ml. of the carbonate had been added and the fermentation was complete. Keeping the sodium carbonate solution in a graduated container allowed a visible record of the rate of consumption of the salt solution to be kept. The rate of consumption corresponded to the rate of fermentation.

The low yield of glycerol is not surprising for this fermentation as the pH was 6.8 for about 20 hours. Also, the addition of

sodium carbonate by dilute solution is not conducive to high glycerol yields according to McDermott (1929) as decreases were general when dilute solutions were used. Solid sodium carbonate was generally used when higher yields were obtained. However, it was the first successful automatically controlled fermentation at a pH as alkaline as 7.5. Toward the completion of the fermentation after 90 hours of incubation and control had occurred, it was found that the controller was no longer needed. No more soda ash was added after that, and the pH gradually increased to 7.9. The fermentation may have been complete in 90 hours.

The use of sodium carbonate with ammonium sulfate in fermentations was described in the discussion concerning the data of Table 24.

d. The use of sodium hydroxide. Since an extremely concentrated solution of sodium carbonate could not be prepared satisfactorily, the dilution of the pH controlled medium described previously was an undesirable result. The low glycerol yield was presumably due to the dilution of the fermenting medium. However, a much more concentrated solution may be made employing sodium hydroxide in place of sodium carbonate. The volume of solution required for automatic pH control would be much less using concentrated sodium hydroxide both because of the higher concentration possible and because of the greater alkalizing capacity of the pure base.

An automatic pH control was attempted on 3 liters of optimum

medium where the sodium hydroxide solution employed for pH control had a concentration of 0.5 gm. per ml. The first attempt ended in failure when a relay broke and all of the base in the reservoir emptied into the fermenting medium. Fermentation ceased.

The second attempt was successful. There were prepared 1.5 liters of optimum semi-synthetic medium containing 15 percent dextrose. Sterilization was for 20 minutes at 15 pounds steam pressure. Inoculation was with 200 ml. of a 48 hour culture of yeast No. 43. The inoculum medium was initially 10 percent in dextrose. The incubation temperature was 30^{0} centigrade. Over a period of six hours after inoculation, the pH was gradually increased to 8 by hand. The pH control was then set at pH = 8. At intervals the control was cut out and the decrease in pH as shown on the record was an indication of fermentative activity. Very little mechanical difficulty was encountered. After about 80 hours of fermentation, the rate of base consumption fell off indicating that the fermentation was nearly completed. At 118 hours the control was stopped and the medium was analyzed. The volume of base added automatically was 175 ml. The total volume of resulting medium was 1875 ml., accounting for both the inoculum and added basic solution. The dextrose concentration initially, calculated on the basis of the final volume, was 12.3 gm. per 100 ml. of medium. Analysis showed 2.5 percent residual dextrose and a glycerol yield of 22.8 percent on the sugar weight. This was practically twice the glycerol yield found in the pH controlled fermentation using a sodium carbonate solution for control.

4. The sulfite-bisulfite dissimilations of dextrose for the preparation of glycerol.

a. The use of ammonium sulfite. The use of ammonium sulfite and of ammonium bisulfite had not been mentioned in literature, previous to a note in the analytical paper of Fulmer, Hickey, and Underkofler (1940). Water solutions of ammonium sulfite are not as alkaline as are water solutions of sodium sulfite, though they are alkaline.

The use of ammonium sulfite and bisulfite was considered as a possibility for the sulfite fermentation as the added salts would be rather easily removed, presumably. The contemplated salt removal method would consist of the addition of calcium oxide or calcium hydroxide to the fermented medium thus precipitating calcium sulfite. Filtration would remove the precipitate, and carbon dioxide could be used to remove the excess lime. Distillation of the filtrate would remove volatile material including ammonia which might be recovered and reused. The residue should be a glycerol syrup containing mainly nutrient salts unless a saccharinic material such as molasses was used. Raw materials might be molasses, pure sugars, or hydrols, ammonia, sulfur dioxide, and calcium oxide or hydroxide. The ammonia and sulfur dioxide might be used cyclicly if efficient enough recovery methods were obtained. Automatic pH control using sulfur dioxide as the controlling chemical would be desirable. The fermenting medium becomes more alkaline as fermentation proceeds since the

bisulfite becomes tied to the aldehyde and ammonium carbonate or bicarbonate result, thus increasing the pH. Hence, sulfur dioxide would be used in greater amounts than would ammonia.

For preliminary experiments ammonium sulfite monohydrate was prepared by running sulfur dioxide gas into concentrated ammonium hydroxide solution until the ammonia odor disappeared. Then enough ammonium hydroxide was added to again bring about the odor of ammonia. This precaution was to remove the possibility of bisulfite formation.

TABLE 26

ACCLIMATIZATION OF INOCULUM WITH AMMONIUM SULFITE, OBSERVING pH

Medium: One 250 ml. flask containing 150 ml. of optimum medium, 10% in dextrose.
Inoculum: 10 ml. of culture No. 43 in 5% dextrose medium.
Incubation: 75 hours at 30° C.
Ammonium sulfite added: $(NH_4)_2SO_3 \cdot H_2O$ added as a solid; 5 gm. added over a period of 28 1/2 hours in 1 gram portions.

Time	27 hr.	46 1/2 hr.	74 hr.
pH	6.12	6.18	6.47
Growth	good	good	good

The addition of ethyl alcohol to the chilled mixture resulted in an excellent yield of pure white crystals which were filtered and dried. This salt was prepared since none was at hand and the preparation was simple.

In order to acclimatize the yeast culture No. 43 to the soluble

ammonium sulfite, as was recommended by Muzzey (1932), a flask of optimum medium was prepared and treated as described in Table 26. It was found to be necessary to add the sulfite in small quantities, as the addition of the entire amount in one addition generally stopped all growth. It may be noted that pH increased with time according to the experiment of Table 26. A very active growth was noted throughout.

The above medium was used as an inoculum for four flasks which were to contain varying amounts of ammonium sulfite. To each flask containing 300 ml. of 15 percent dextrose medium was added 10 grams of ammonium sulfite. The pH of each flask was measured before inoculation and was found to be 7.44. Then 30 ml. of the inoculum were added to each flask; the pH was then found by glass electrode to be 7.38. At the end of four days of incubation at 30° centigrade there was found to be practically no fermentation. Growth had ceased.

This experiment was attempted before the inhibiting effects of ammonia under alkaline pH conditions were noted as described in the ammonium hydroxide section. However, it is useful as further proof of the inhibiting characteristics of molecular ammonia or ammonium hydroxide in solution. Further work remains to be done on fermentations with ammonium sulfite to prove the value of such a fermentation. However, it was shown that yeast will ferment dextrose in the presence of this sulfite.

b. The use of calcium and magnesium sulfites. The use of calcium and magnesium sulfites for acetaldehyde fixation in fermentations had been noted by Neuberg and Reinfurth (1919). They also made a few studies using zinc sulfite, but the results using this reagent were not promising. They found that agitation induced increased acetaldehyde fixation during fermentations in the presence of slightly soluble sulfites. They also found that the use of freshly precipitated calcium sulfite in fermentations resulted in higher acetaldehyde fixation than did the use of the commercial anhydrous salt. The studies of Neuberg and Reinfurth were designed to give further evidence concerning the proof of the sugar dissimilation mechanism. Acetaldehyde was apparently the main product being studied. Nothing was indicated as to possible industrial application for the production of glycerol.

Two experiments were run by Neuberg and Reinfurth where attempts were made to adjust the acidity of the fermenting media in the presence of calcium sulfite. In one experiment, the acid reagent used was primary potassium phosphate; in the other experiment the acid reagent was phosphoric acid. The organism used for fermentation was a top yeast. The fermentations were completed in about 26 and 42 days respectively. The acetaldehyde found was 14.86 percent and 15.22 percent of the hexose weight, respectively. A purpose of these experiments was to show that acetaldehyde fixation occurred in acid media. Such methods would not be industrially practical because of

the long time involved.

The extensive employment of reagents such as calcium and magnesium sulfites for fermentations producing glycerol has not been reported. The use of such reagents would have the advantage of easy removal from fermentation media in contrast to the difficulties involved when sodium sulfite and bisulfite are used. It is generally acknowledged that the bisulfite ion is necessary for acetaldehyde fixation in these fermentations, and it is rather uncertain what the bisulfite ion concentration in solution would be if an excess of calcium or magnesium sulfite were added to a fermentation medium. The solubility of the sulfite would be an important factor to consider, as well as the effect of acids formed by fermentation on the solubility of the sulfite. The effect of carbon dioxide on the salt would be expected to be rather small in slightly acid solutions since sulfurous acid is a stronger acid than is carbonic acid. It is obvious that decreasing the pH of the mixture would increase the bisulfite ion concentration. The ideal situation would be such that there would be available sufficient bisulfite ions to fix all of the acetaldehyde and still not enough to exhibit toxic characteristics. An approach toward the ideal might be made by pH control using sulfur dioxide as the pH controlling agent in the presence of calcium or magnesium sulfite. An acid pH should be maintained. The ideal pH values would be expected to be somewhat different for calcium and magnesium sulfites because of the effect of their different solubilities on the bisulfite ion concentration. The solubilities of calcium and

magnesium sulfites are given in Table 27.

TABLE 27

SOLUBILITIES OF CALCIUM AND MAGNESIUM SULFITES AND BISULFITES

Salt	Solubility in Parts per 100 Parts H_2O
$CaSO_3 \cdot 2H_2O$	0.0043 at 18° C.
$CaSO_3 \cdot 2H_2O$	0.0027 at 90° C.
$MgSO_3 \cdot 6H_2O$	1.25 cold
$MgSO_3 \cdot 6H_2O$	0.833 hot
$Ca(HSO_3)_2$	soluble; exists in solution only.
$Mg(HSO_3)_2$	soluble; exists in solution only.
	Solubility in sugar solutions
$CaSO_3$	0.0825 gm. in 1 liter 10% sugar soln.
$CaSO_3$	0.800 gm. in 1 liter 30% sugar soln. (18° C.)
0.533 gm. $CaSO_3$ dissolves in 100 cc. H_2O containing 9 gm. SO_2.	

(Adapted from Mellor (1930))

A method for glycerol production utilizing sulfur dioxide in connection with calcium or magnesium sulfites would be related in a way to the method of Barbet (1928) who used only sulfur dioxide (or sulfurous acid) in fermentations. Free sulfurous acid would be rather difficult to use, probably, because of the toxicity of high bisulfite concentration. If the concentration were kept low enough to be non-toxic, then there would most probably be low acetaldehyde fixation. The lack of extensive industrial utilization of the method of Barbet might be a point of evidence supporting this contention. The volatility of sulfur dioxide would be an advantage in glycerol

recovery compared with the methods necessary when sodium salts are used.

The solubilities of calcium and magnesium sulfites indicated in Table 27 were not given as functions of pH. It was deemed desirable to determine the solubilities of these salts as functions of pH, since they are to be used subsequently under various pH conditions.

The solubility of both Merck's anhydrous calcium sulfite and freshly precipitated calcium sulfite were studied as pH functions at 25° centigrade. Saturated aqueous solutions were prepared where the pH values were adjusted by means of sulfuric acid. The mixtures

TABLE 28

SOLUBILITIES OF HYDRATED AND ANHYDROUS CALCIUM SULFITES AS FUNCTIONS OF pH

Anhydrous $CaSO_3$			Hydrated $CaSO_3$		
pH	Ml. of 0.1 N I_2 Solution Consumed by 10 ml. of Sulfite Solution.	Grams per 100 ml. of Dissolved $CaSO_3$ (calculated)	pH	Ml. of 0.1 N I_2 Solution Consumed by 10 ml. of Sulfite Solution	Grams per 100 ml. of Dissolved $CaSO_3$ (calculated)
3.55	16.6	0.996	3.61	16.71	1.002
3.78	12.44	0.746	3.95	9.71	0.583
4.00	8.97	0.538	4.31	5.04	0.302
4.12	7.44	0.446	4.81	2.56	0.154
4.29	5.86	0.352	5.45	1.08	0.065
4.49	4.50	0.270	5.81	0.48	0.029
4.62	3.89	0.233	6.44	0.28	0.017
4.91	2.20	0.132	7.68	0.04	0.002
5.42	0.98	0.059	-	-	-
6.42	0.15	0.009	-	-	-
7.52	0.05	0.003	-	-	-

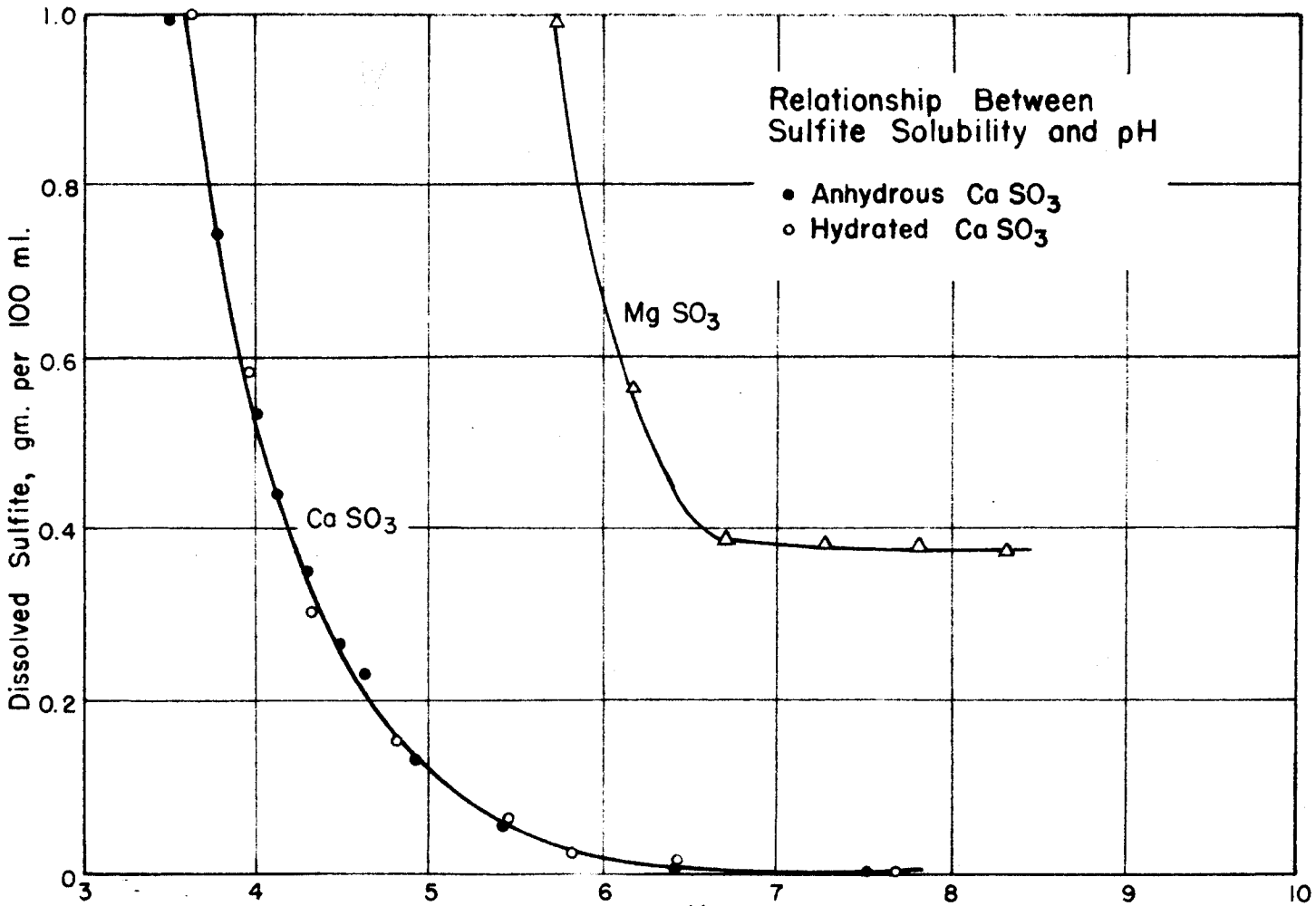
were shaken at intervals over a period of ten hours. The pH values were determined by means of the Cameron pH Meter. The dissolved sulfite was determined volumetrically by titrating 10 ml. samples of the clear solutions with a 0.1 normal iodine solution. Starch solution was used as an indicator. On milliliter of the standard iodine solution is equivalent to 0.0060 gram of calcium sulfite according to the equation:

$$CaSO_3 + I_2 + H_2O \longrightarrow CaSO_4 + 2HI$$

The data for the solubilities of calcium sulfite are given in Table 28 and in Figure 9.

The solubility of magnesium sulfite was measured as a function of pH in a manner analogous to the methods used for calcium sulfite determinations. Sulfuric acid was used for pH adjustments, and the pH was measured at 25° centigrade as before. One milliliter of 0.1 normal iodine solution is equivalent to 0.0052 gram of magnesium sulfite ($MgSO_3$). Experimental solubility data are given in Table 29 and in Figure 9.

The results shown in Tables 28 and 29 and in Figure 9 show a sharp increase in the solubility of calcium sulfite as the pH decreases below six, and in a similar increase in the solubility of magnesium sulfite was noted at about pH = 6.5. It was considered that as the pH went below these values, increasing acetaldehyde fixation would occur along with increased toxicity. Data are given


Relationship Between
Sulfite Solubility and pH
• Anhydrous Ca SO3
○ Hydrated Ca SO3
Mg SO3
Ca SO3
Dissolved Sulfite, gm. per 100 ml.
1.0
0.8
0.6
0.4
0.2
0
3
4
5
6
7
8
9
10
pH
Figure 9

subsequently related to this consideration.

It was thought that perhaps the presence of acetaldehyde would increase the solubility of calcium sulfite because of the formation of the acetaldehyde-bisulfite complex. Some quantitative data concerning such a solubility relationship to pH was desired. Such reactions occur in fermentation media, and it was hoped that some

TABLE 29

THE SOLUBILITY OF MAGNESIUM SULFITE AS A FUNCTION OF pH

pH	Ml. of 0.1 N. I_2 Solution Consumed by 10 ml. of Sulfite Solution	Grams per 100 ml. of Dissolved $MgSO_3$. (calculated)
5.35	21.60	1.122
5.72	19.08	0.992
6.16	10.83	0.564
6.70	7.39	0.384
7.27	7.36	0.383
7.81	7.29	0.380
8.32	7.28	0.379

information might be obtained which would be of value in fermentations for glycerol production.

Studies were made with saturated hydrated calcium sulfite solutions in the presence of a fixed concentration of acetaldehyde but with varying pH levels. Aqueous solutions containing an excess of solid calcium sulfite were prepared. These solutions were adjusted to various pH levels by means of sulfuric acid solution. Acetaldehyde

was added to these solutions as an aqueous solution so that there was as final concentration 0.77 gm. of acetaldehyde per 100 ml. of solution. On the addition of the acetaldehyde the pH of the solutions increased markedly. The pH rose in one solution from 3.6 to 8.5, and from 7.68 to 10.5 in another solution. The acetaldehyde undoubtedly combined with the available bisulfite ions thus causing an increase in the pH.

The free sulfite was determined by direct iodine titration, and the combined sulfite was determined by further iodine titration upon the addition of excess solid sodium bicarbonate to the solution. Data are given in Table 30 and in Figure 10.

The results of Table 30 and Figure 10 indicate a great increase in the solubility of calcium sulfite in the presence of acetaldehyde as compared to its solubility in water (see Table 28). It may be noted, especially in Figure 10, that the acetaldehyde-bisulfite complex commences to decompose with increasing rapidity as the pH increases past 4.5. This fact is indicated by the decrease in fixed sulfite with an increase in pH past 4.5.

A preliminary pH recording was made on 1.585 liters of 15 percent dextrose medium with 160 gm. of Merck's anhydrous calcium sulfite added. Inoculum was 150 ml. of 10 percent dextrose culture of yeast No. 43. The initial pH was 6.6. At 24 hours the pH was 4.5 and at 48 hours the pH was 5. At about 57 hours there was a sharp rate of pH change and pH = 6.5 was shortly reached. At 96 hours

the pH was 6.9. Analysis showed 0.8 percent residual dextrose and a yield of 8.81 percent of the dextrose weight as glycerol. When the pH was 7 or more, the odor of acetaldehyde was quite apparent.

TABLE 30

SOLUBILITY OF CALCIUM SULFITE AS A FUNCTION OF pH IN THE PRESENCE OF A CONSTANT CONCENTRATION OF ACETALDEHYDE

pH	Ml. of 0.1 N. I_2 for Free Sulfite in 10 ml. of Solution	Ml. of 0.1 N. I_2 for Total Sulfite in 10 ml. of Solution	Ml. of 0.1 N. I_2 for Combined Sulfite in 10 ml. of Solution	Free Sulfite as $CaSO_3$. Gm. per 100 ml.	Combined Sulfite as $CaSO_3$. Gm. per 100 ml.
1.21	1.52	27.93	26.41	0.091	1.59
3.22	1.86	28.05	26.19	0.112	1.57
3.64	1.67	28.02	26.35	0.106	1.58
4.19	1.72	28.72	27.00	0.103	1.62
4.33	1.75	28.43	26.68	0.105	1.60
4.85	0.62	27.05	26.43	0.037	1.59
5.38	0.21	26.52	26.31	0.013	1.58
6.40	0.21	24.87	24.66	0.013	1.48
6.71	0.19	22.83	22.64	0.011	1.36
7.18	0.15	21.27	21.12	0.009	1.27
7.60	0.12	13.21	13.09	0.007	0.79
8.60	0.06	8.48	8.42	0.004	0.51

Experiments were run to check the statements of Neuberg and Reinfurth (1919) that freshly prepared calcium sulfite was better for acetaldehyde fixation than was the commercial anhydrous salt. The fermentations were conducted at various pH values to see if there were variations in glycerol production at different pH levels for the different types of calcium sulfite.

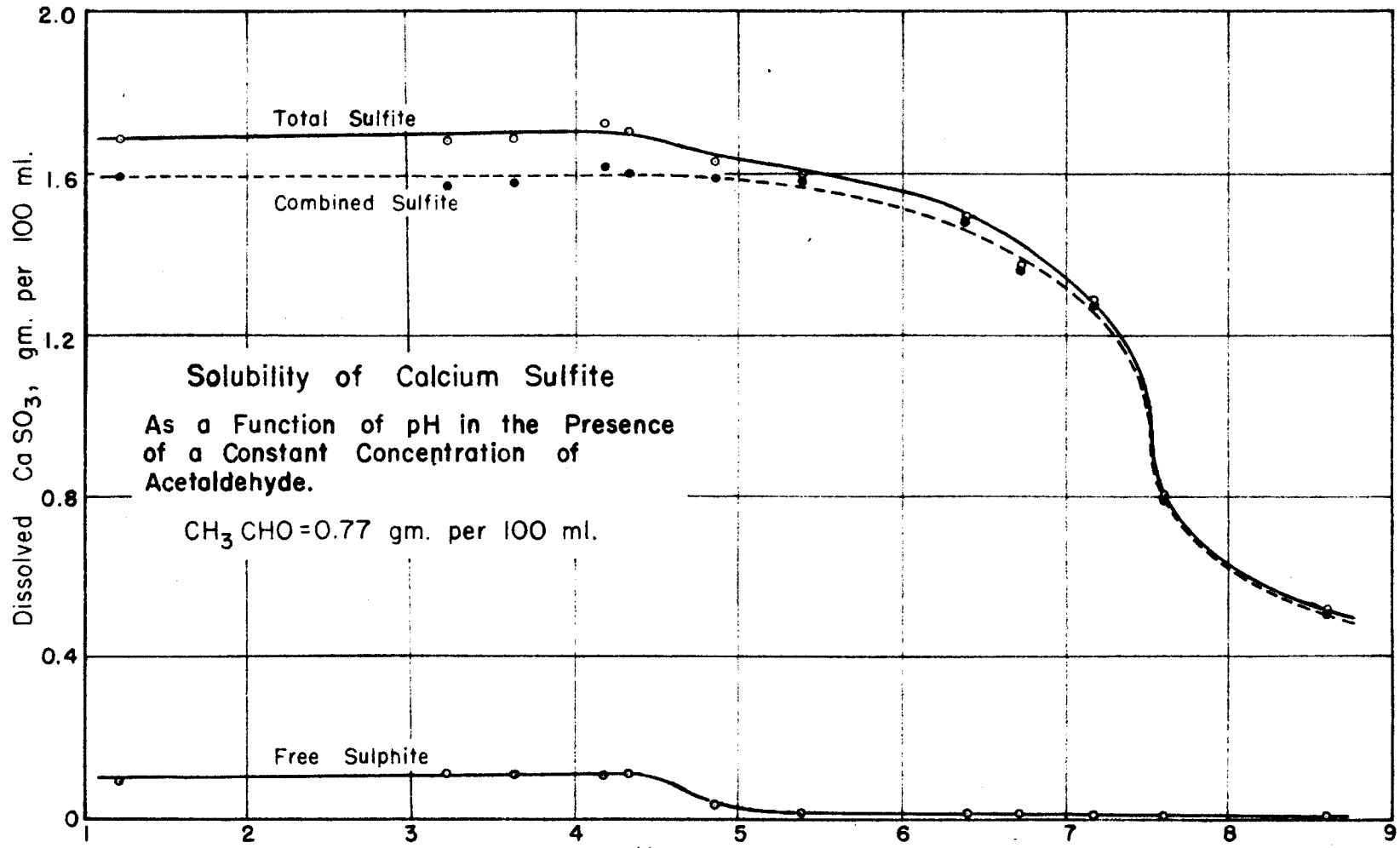

Total Sulfite
Combined Sulfite
Solubility of Calcium Sulfite
As a Function of pH in the Presence of a Constant Concentration of Acetaldehyde.
CH_3CHO = 0.77 gm. per 100 ml.
Free Sulphite
Dissolved $CaSO_3$, gm. per 100 ml.
2.0
1.6
1.2
0.8
0.4
0
1
2
3
4
5
6
7
8
9
pH

Figure 10

Optimum semi-synthetic medium was prepared containing 15 percent dextrose. Two hundred ml. of this medium were placed in each of 10 Erlenmeyer flasks of 300 ml. capacity and sterilized in the usual way. Twenty ml. of 10 percent dextrose inoculum of yeast No. 43 were added to each flask. Calcium sulfite was added as shown in Tables 31 and 32. The increased solution volumes in Table 32 over the volumes in Table 31 were due to the fact that the added calcium sulfite of Table 32 was damp. Incubation was for four

TABLE 31

EFFECT OF pH ON GLYCEROL YIELD AND ON SUGAR CONSUMPTION IN FERMENTATIONS IN THE PRESENCE OF ANHYDROUS CALCIUM SULFITE

pH	Total Dextrose Including Inoculum, Grams	Weight of $CaSO_3$ per Flask, Grams	Final Volume Exclusive of Solid, Ml.	Percent Residual Dextrose	Glycerol Found	
					% on Dextrose Consumed	% on Total Dextrose
4.0	33	20	220	80.6	24.7	4.79
4.5	33	20	220	3.06	11.00	10.66
5.0	33	20	220	0.94	10.31	10.20
5.5	33	20	220	0.54	10.68	10.68
6.0	33	20	220	0.54	10.39	10.31

days at 30° centigrade. The flasks were frequently shaken by hand. The pH was adjusted at intervals by sulfuric acid additions.

The results indicate that anhydrous calcium sulfite is not as effective as is the hydrated salt in acetaldehyde fixation. These observations verify the observations of Neuberg and Reinfurth (1919). It was also observed that decreasing pH in the range studied using

anhydrous calcium sulfite did not appreciably affect the glycerol yield of experiments in which the sugar was consumed fairly completely. However, when the hydrated salt was used, an increase in glycerol yield was noted with decreasing pH.

TABLE 32

EFFECT OF pH ON GLYCEROL YIELD AND ON SUGAR CONSUMPTION IN FERMENTATIONS IN THE PRESENCE OF HYDRATED CALCIUM SULFITE

pH	Total Dextrose Including Inoculum, Grams	Weight of $CaSO_3$ per Flask, Anhydrous Basis, Grams	Final Volume, Exclusive of Solid, ML.	Percent Residual Dextrose	Glycerol Found % on Dextrose Consumed	Glycerol Found % on Total Dextrose
4.0	33	20	245	84.0	27.8	4.45
4.5	33	20	245	86.0	26.4	3.70
5.0	33	20	245	0.89	14.23	14.1
5.5	33	20	245	0.86	12.38	12.27
6.0	33	20	245	0.594	11.39	11.31

It is very probable that continuous agitation and the use of larger quantities of calcium sulfite would have increased the glycerol yields.

In the experiments described in Tables 31 and 32, it is noteworthy that a sharp decrease in the sugar consumption occurred as the pH went from 5 to 4.5 and below. Bisulfite ion toxicity was no doubt the cause of these results.

A multiple stirrer was acquired which was capable of stirring the media of six flasks simultaneously and continuously. A disadvantage of the stirrer was the fact that it could not be placed

in an incubator very readily, so the fermentations were run at room temperature which was usually about 26° centigrade instead of 30° which was the incubator temperature.

A fermentation series was prepared in which 118 gm. of moist hydrated calcium sulfite (equivalent to 60 gm. of anhydrous salt) were added to the fermentation flasks each of which contained 200 ml. of 15 percent optimum medium. The inoculum was 20 ml. of a 48 hour culture of yeast No. 43 grown in 10 percent dextrose medium; the pH values were 4.7, 4.8, 4.9, 5.0, and 5.5. These values were chosen since they seemed to be close to the critical pH range. The most acid fermentation possible was considered desirable in order to have maximum bisulfite concentration for maximum acetaldehyde fixation. The media were continuously stirred at about 26° centigrade. Fermentations were unsuccessful where the pH was below 5. Lack of acclimalization of the culture to the above conditions was considered responsible for the lack of growth in the media more acid than pH = 5. However the fermentations at pH = 5.0 and 5.5 resulted in glycerol yields of 15.1 and 12.2 percent on the sugar weight, respectively. Residual sugar was approximately 0.5 percent of that initially present. Since so many members of the series did not ferment, the results were not especially conclusive. It was observed that the medium at pH = 5.0 did not ferment well for about the first 48 hours after inoculation as compared to the immediate fermentation when the pH was 5.5. However, once the fermentation started at pH = 5, it

proceeded to completion smoothly. This observation might be considered evidence that unacclimatized yeast will grow in such a medium if given sufficient time to get used to the conditions. The pH was adjusted when necessary with 18 normal sulfuric acid. The volume required per flask was negligible.

It was considered as possibly desirable to filter the solid calcium sulfite and yeast cultures from the previously fermented media and to use the resulting cake as inoculum for a subsequent series of fermentations. Such a culture in direct contact with calcium sulfite would surely be acclimatized to the sulfite to some extent. The cake containing the culture was always kept damp.

A series of five flasks was prepared containing 200 ml. of optimum medium. Sterilization of the medium was not considered necessary. Each flask was inoculated with one-fifth of the cake from the two successfully fermented flasks described above. Additional hydrated calcium sulfite was added such that the total weight of the salt per flask was equivalent to 60 gm. of the anhydrous salt. The pH values studied were near the critical pH range where the bisulfite ion concentration begins to rise rapidly with decreasing pH. The results of the experiment are given in Table 33.

From the data of Table 33 one may conclude that the glycerol yield approaches a constant value of about 10 percent of the dextrose weight practically independent of pH variations under the conditions described above.

The following equilibrium was considered:

$$Ca(HSO_3)_2 + 2CH_3CHO \rightleftharpoons Ca[HSO_3 \cdot CH_3CHO]_2$$

The rate of decomposition of the acetaldehyde complex would be independent of the concentrations of calcium bisulfite and acetaldehyde. The rate of formation, however, would be dependent upon the concentrations of these compounds. As the concentration of the complex builds

TABLE 33

GLYCEROL YIELDS FROM YEAST FERMENTATIONS AT DIFFERENT pH LEVELS USING CALCIUM SULFITE CAKE FROM PREVIOUS FERMENTATIONS AS INOCULUM

pH	Filtered Volume, ml.	Initial Dextrose, gm. per 100 ml.	Residual Dextrose %	Glycerol Found	
				Gm. per 100 ml.	% on Dextrose
5.3	208	18.22	0.42	1.94	10.06
5.1	200	18.95	0.50	1.92	10.01
4.9	200	18.95	0.52	1.95	10.03
4.7	200	18.95	0.52	2.00	10.05
4.5	200	18.95	High;	fermentation ceased.	

up, the rate of decomposition would increase thus allowing a greater concentration of acetaldehyde to be present thus allowing its greater enzymatic reduction to ethanol and a corresponding reduction in the glycerol yield. If these suppositions are true, then decreasing the initial sugar concentrations in the media should result in increased percentage glycerol yields. The lower sugar concentrations

should give less possible acetaldehyde, theoretically, and thus the concentration of the aldehyde-bisulfite complex would be lower in solution thus allowing a greater percentage of the acetaldehyde to be fixed. This would result in higher glycerol yields.

A series of media was prepared to study the relationship between the initial dextrose concentration of the medium and the glycerol yield in the presence of hydrated calcium sulfite. The inoculum was the filtered solid calcium sulfite cake containing the yeast No. 43 of previous fermentations. The calcium sulfite present per flask was equivalent to 60 gm. of the anhydrous salt. The media were continuously stirred at 26° centigrade. The fermentation time decreased with decreasing initial dextrose concentration; however, all media were allowed to stand for four days before analyses were run. The pH was not controlled accurately in the media, but all members of the series were treated alike. The results of this investigation are given in Table 34. The implications may be more clearly observed in Figures 11 and 12.

The data of Table 34 prove conclusively that the percentage glycerol yield increases with decreasing initial dextrose concentration under the previously described conditions. A corollary to this conclusion is that the percent of acetaldehyde fixation decreases with increasing initial dextrose concentration. This evidence supports rather strongly the contentions described above concerning the adetaldehyde-bisulfite equilibrium. The acetaldehyde-bisulfite

complex concentration very likely approaches a maximum under a specified set of fermentation conditions. A mathematical relationship between the initial dextrose concentration and the glycerol yield is presented following Table 37.

Following the investigations on fermentations in the presence of calcium sulfite, it was decided to run some yeast fermentations in

TABLE 34

THE EFFECT OF DEXTROSE CONCENTRATION ON THE GLYCEROL YIELD OF FERMENTATIONS IN THE PRESENCE OF CALCIUM SULFITE

pH	Initial Dextrose, gm. per 100 ml.	$CaSO_3$ Present, Gm. Dry Basis	Filtered Medium Volume, ml.	Residual Dextrose, gm. per 100 ml.	Glycerol Yield	
					Gm. per 100 ml.	% on Dextrose
5.5-5.1	4	60	200	0.084	0.83	20.8
5.5-5.0	8	60	200	0.084	1.32	16.5
5.5-5.1	12	60	200	0.056	1.51	12.6
5.5-5.0	16	60	200	0.096	1.56	9.8

the presence of magnesium sulfite ($MgSO_3 \cdot 6H_2O$) with special attention being paid to pH, glycerol yield, and initial sugar concentration.

Two preliminary fermentations were run using magnesium sulfite in place of calcium sulfite. The technique employed was the same as was used in the fermentations involving calcium sulfite. It is of importance that magnesium sulfite is somewhat more soluble than is calcium sulfite. Solubility data are given in Table 27. The greater solubility of magnesium sulfite should result in a higher acetaldehyde

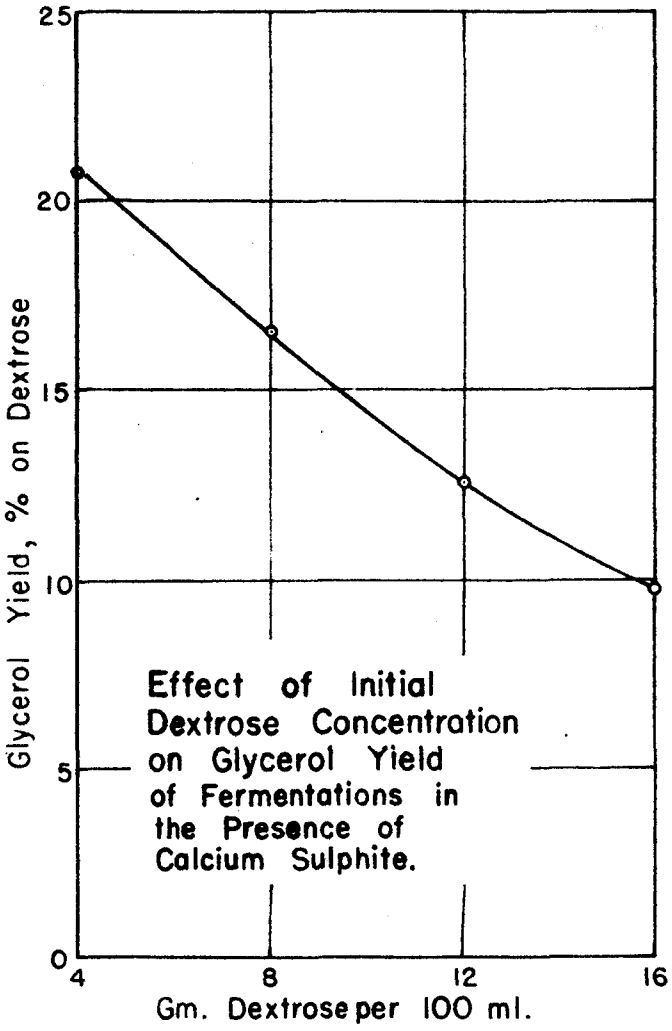


Figure 11

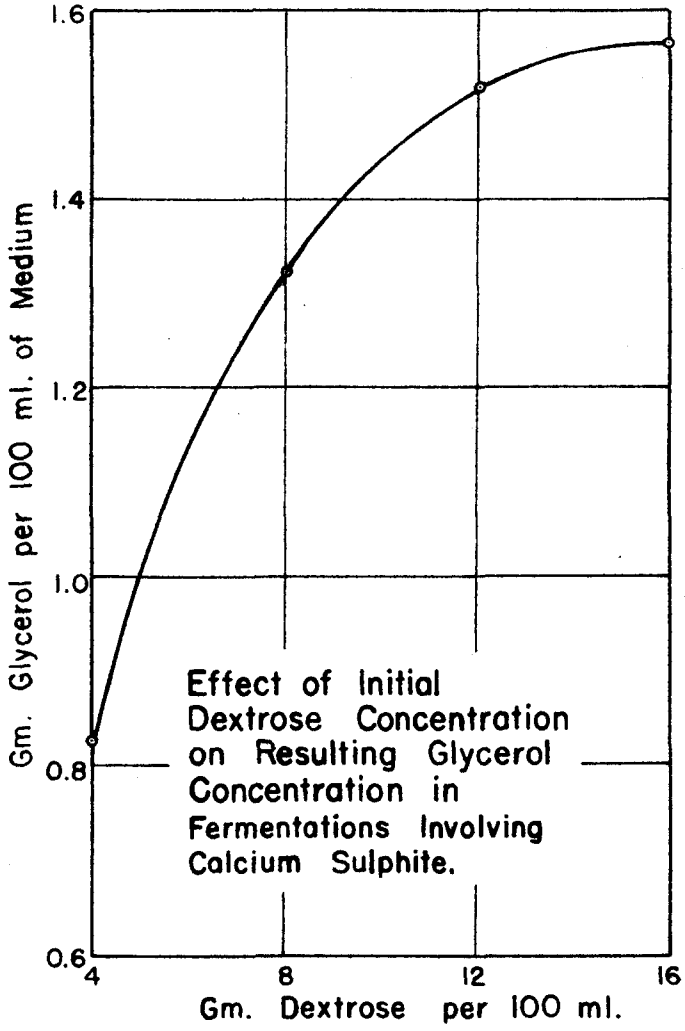


Figure 12

fixation than occurred when calcium sulfite was used; hence, a greater glycerol yield should be obtained. The inoculum used was a filtered calcium sulfite cake as described previously. The initial unadjusted pH of both media after magnesium sulfite was added was about seven. On fermenting about 24 hours the pH dropped to about 6.3. At about

TABLE 35

PRELIMINARY FERMENTATIONS IN THE PRESENCE OF MAGNESIUM SULFITE WITH pH UNADJUSTED

pH Range	Initial Dextrose, gm. per 100 ml.	$MgSO_3 \cdot 6H_2O$ Present, gm.	Volume of Medium ml.	Residual Dextrose, gm. per 100 ml.	Glycerol Found	
					Gm. per 100 ml.	% on Dextrose
7.1-6.3	8	50	200	0.048	1.765	22.05
7.1-6.2	8	50	200	0.076	1.777	22.2

50 hours the pH had increased to 7.1 and the media were quiescent. Analyses were run four days after inoculation. No attempt was made to control the pH in the media. Results are given in Table 35.

The results of Table 35 indicate that magnesium sulfite is an efficient acetaldehyde fixing agent since about 22 percent glycerol was found. This value is about 45 percent of the theoretical yield. The fermentation time was but a fraction of the time required in the experiments of Neuberg and Reinfurth (1919). These data prove that magnesium sulfite is a more efficient acetaldehyde fixing agent than is calcium sulfite. Greater glycerol yields resulted, thus verifying the previous prediction.

It was desired to study the effect of pH on the yield of glycerol in fermentations in the presence of magnesium sulfite. The inoculum was the filtered magnesium sulfite cake containing yeast No. 43 from the fermentations of Table 35. The media were not sterilized. The antiseptic action of the magnesium sulfite in solution was sufficient to keep contamination at a low level. The pH of each medium was adjusted by means of frequent additions of several drops of 7.5 normal sulfuric acid. The media were stirred continuously. The temperature was about 26° centigrade. Vigorous fermentations were noted. The fermentation time was four days. Results are given in Table 36.

TABLE 36

THE RELATION OF pH TO THE GLYCEROL YIELD OF FERMENTATIONS IN THE PRESENCE OF MAGNESIUM SULFITE

pH Range	Filtered Volume, ml.	Total Weight $MgSO_3 \cdot 6H_2O$ gm.	Initial Dextrose, gm. per 100 ml.	Final Dextrose, gm. per 100 ml.	Glycerol Found	
					Gm. per 100 ml.	% on Dextrose
5.5	206.4	60	9.68	0.14	2.25	23.3
5.8	207.4	60	9.66	0.14	2.235	23.1
6.0	205.6	60	9.75	0.12	2.185	22.4
6.3	204.5	60	9.78	0.13	2.20	22.5
6.5	203.0	60	9.86	0.13	2.24	22.7
7.0	201.0	60	9.95	0.13	2.37	23.8

The results of Table 36 indicate that pH is not a critical function in the range studied and under the conditions employed. These results are similar to the corresponding results in the studies with calcium sulfite.

The next investigations were concerned with the effect of inital dextrose concentration on the glycerol yield of fermentations in the presence of magnesium sulfite. The theoretical considerations described previously for the use of calcium sulfite in fermentations apply qualitatively in the investigations with magnesium sulfite. The optimum

TABLE 37

THE EFFECT OF DEXTROSE CONCENTRATION ON THE GLYCEROL YIELD OF FERMENTATIONS IN THE PRESENCE OF MAGNESIUM SULFITE

Final Filtered Volume, ml.	Weight of $MgSO_3 \cdot 6H_2O$ per Flask, gm.	Initial Dextrose, gm. per 100 ml.	Time to Quiescence, hours	Final Dextrose, gm. per 100 ml.	Glycerol Found	
					Gm. per 100 ml.	% on Dextrose
220	40	4.54	15	0.127	1.079	23.8
220	40	9.08	30	0.080	2.032	22.4
220	40	13.62	48	0.080	2.780	20.4
223	40	17.94	>72	0.121	3.320	18.5
220	40	22.70	>72	0.126	3.985	17.6

semi-synthetic medium was used as a basis for these fermentations as in previous experiments. The inoculum was an acclimatised culture of yeast No. 43 grown in the presence of magnesium sulfite without pH adjustments. Inoculation was 20 ml. of a 48 hour culture of this yeast grown in a 10 percent dextrose medium. The temperature was about 27° centigrade. Continuous stirring was maintained. The pH values of the media were uncontrolled. In all flasks the pH was initially about seven; at 48 hours the pH was about 6.3, and at four days the pH had again returned to seven. The experimental data are given in

Table 37.

The results of Table 37 indicate that increasing the initial dextrose concentration causes an increase in the final glycerol concentration and a decrease in the percentage yield of glycerol. This

TABLE 38

THE RELATIONSHIP BETWEEN EXPERIMENTAL AND CALCULATED YIELDS OF GLYCEROL FROM FERMENTATIONS INVOLVING CALCIUM SULFITE

Initial Dextrose, Gm. per 100 ml.	Glycerol Yield, Percent on Dextrose	
	Experimental	Calculated
4	20.8	20.4
8	16.5	16.0
12	12.6	12.5
16	9.8	9.8

trend is quite similar to that found for fermentations in the presence of calcium sulfite.

A mathematical consideration of the data of Tables 34 and 37 shows the percent yield of glycerol, G, to be an exponential function of the initial concentration of dextrose, D, that is,

$$\log G = m D + \log k$$

The equations for the calcium sulfite and magnesium sulfite are, respectively:

$$\log G_1 = -0.0267 D + 1.417$$

$$\log \underline{Q}_2 = -0.00730\ \underline{D} + 1.417$$

An extrapolation indicates the maximum yield, at a dextrose concentration of zero grams per 100 ml., to be about 26 percent under the conditions of the experiments. However, whether such an extrapolation is entirely justified would need to be supported by additional data in the lower ranges of concentration of dextrose.

The agreement between the experimental values and those calculated by means of the above equations is shown in Tables 38 and 39.

TABLE 39

THE RELATIONSHIP BETWEEN EXPERIMENTAL AND CALCULATED YIELDS OF GLYCEROL FROM FERMENTATIONS INVOLVING MAGNESIUM SULFITE

Initial Dextrose, Gm. per 100 ml.	Glycerol Yield, Percent on Dextrose	
	Experimental	Calculated
4.54	23.8	24.2
9.08	22.4	22.4
13.62	20.4	20.8
17.94	18.5	19.3
22.70	17.6	17.8

Data were desired concerning the success of automatic pH-controlled fermentations in the presence of solid magnesium sulfite. Media were prepared and the pH was automatically controlled by an acetic acid solution (50 percent by volume). The inocula were cultures of S. cerevisiae No. 43. The starting media were 1.5 liters of optimum media containing 15 percent dextrose. Fermentation temperature was

30° centigrade. About 280 gm. of magnesium sulfite were added to each batch. Experimental data are given in Table 40.

TABLE 40

GLYCEROL YIELDS FROM FERMENTATIONS WITH AUTOMATICALLY CONTROLLED pH IN THE PRESENCE OF MAGNESIUM SULFITE

pH	Inoculum	Ml. of Acetic Acid Added	Total Volume Excluding Solids ml.	Initial Dextrose, gm. per 100 ml.	Residual Dextrose, gm. per 100 ml.	Glycerol Found	
						Gm. per 100 ml.	% on Dextrose
6.5	195 ml. 15% dextrose	60	1755	12.82	0.124	3.04	23.4
6.2	$MgSO_3$ cake	65	1565	14.38	0.070	3.325	23.2

The results of the investigations of Table 40 substantiate those of previous fermentations in the presence of magnesium sulfite insofar as the glycerol yield is concerned. The yields of glycerol using pH control at an acid level appear slightly higher than those of uncontrolled fermentations of corresponding initial dextrose concentrations described in Table 37. The differences in yields are not outstanding, however. The use of acetic acid for pH control is not ideal by any means since the magnesium acetate formed would cause difficulties in the glycerol recovery. The use of sulfur dioxide for automatic pH control would have the advantage of simple removal on completion of the fermentation.

One of the proposed advantages of the method of formation of

glycerol by the use of slightly soluble sulfites is the fact that such compounds may be easily precipitated by suitable pH adjustments and subsequently be easily filtered from the fermented liquor. The resulting medium may then be distilled free from solvents and any additional precipitate filtered. With sugar and perhaps nutrients added, the resulting medium could be refermented to build up the glycerol content in order to make its recovery more feasible economically. Such fermentations are described below.

The solvents were distilled from previously fermented media in the presence of enough calcium hydroxide to keep the solution slightly alkaline. The resulting mixtures were then filtered; dextrose was added to the filtrates which were then diluted to their initial volumes before distillation of the solvents. Inoculation was by means of a calcium sulfite cake containing yeast No. 43 from a previous fermentation. Fermentation occurred at about 27^{0} to 30^{0} centigrade with intermittent stirring for about four days. Results are shown in Table 41.

The relatively low yields indicated in Table 41 were probably partially due to non-continuous stirring and possibly to the relatively wide pH range found since pH was not controlled. However, the data show that more than one fermentation can be run on the same basic substrate with comparative ease. The glycerol content of the medium was increased; more efficient methods should increase the glycerol yield significantly.

TABLE 41

GLYCEROL YIELD WHERE TWO SUCCESSIVE FERMENTATIONS ARE RUN ON THE SAME SUBSTRATE IN THE PRESENCE OF CALCIUM SULFITE

pH Range	$CaSO_3$, Dry Basis, gm.	Ml. Filtered Medium	Initial Dextrose, gm. per 100 ml.	Glycerol content, gm. per 100 ml.			Glycerol Yield, % on Dextrose	Residual Dextrose, gm. per 100 ml.
				Initially Present	Total Found	Added by 2nd Ferm.		
5.8-5.1	60	190	12.63	1.693	2.74	1.05	8.31	0.118
5.8-5.1	60	190	12.63	1.926	2.92	1.00	7.92	0.100

It was desired to find whether a second fermentation could also be run using magnesium sulfite in place of calcium sulfite. The initial glycerol concentration of the medium employed was 3.04 gm. per 100 ml. of solution. Two hundred ml. were taken and volatiles were removed as described above where calcium sulfite was used. Dextrose was added so that the resulting medium diluted back to its initial volume would contain 15 gm. per 100 ml. Thirty ml. of yeast culture No. 43 grown in 10 percent dextrose medium were added as inoculum. The net dextrose concentration was then 13.04 gm. per 100 ml. assuming all of the dextrose was gone from the inoculum. The inoculum contained 0.12 gm. of glycerol. The total initial glycerol concentration was 2.69 gm. per 100 ml. The final glycerol content was found to have been 5.05 gm. per 100 ml. Thus, the increase in glycerol content due to the second fermentation was 2.36 gm. per 100 ml. This corresponds to a yield of 18.1 percent on the dextrose weight. Though the conditions were not necessarily ideal, this experiment showed that more than one fermentation could be run on the same basic substrate using magnesium sulfite to build up the glycerol concentration in the medium toward a desirable value.

V. SUMMARY AND CONCLUSIONS

1. The pH of the fermenting media was followed by means of a Cameron pH Recorder employing a glass electrode. Additional circuits and equipment were devised to automatically control the pH at the desired levels.

2. Improvements were made in the reducing-sugar determination method of Somogyi which resulted in more exact and reproducible results.

3. An optimum semi-synthetic medium was developed to serve as a basal medium for studies on the glycerol fermentations. The medium contained a minimum of fixed solids, that is, solids such as nutrient salts which are difficultly removeable from the fermented medium. A minimum of fixed solids was an important requirement inasmuch as large amounts of such materials cause considerable difficulty in subsequent glycerol recovery.

4. Studies were made on the alkaline fermentation of dextrose by means of yeast using ammonium hydroxide as the alkalising agent. It was found that fermentations were unsuccessful when an appreciable ammonium concentration existed in media in which the pH value was above seven. It was concluded that molecular ammonia or ammonium hydroxide in solution was a toxic agent. The toxicity of the molecular

ammonia or ammonium hydroxide is a function of the activity of the molecular compound. Ammonium ions are relatively non-toxic. The activity and thus the toxicity of ammonia or ammonium hydroxide is a function of both pH and the available ammonium concentration. Consequently, since the toxicity increases sharply as pH increases past seven, the alkaline fermentation to prepare glycerol is not very satisfactory, since greater alkalinity is necessary for high glycerol yields. The advantage of the use of ammonium hydroxide for pH control of fermentations would have been its simple removal following fermentation.

5. Yeast will ferment dextrose in the presence of ammonium sulfite provided the pH of the medium is sufficiently below seven to obviate the toxic effects of ammonia. Acclimatization of the culture to ammonium sulfite is necessary. Ammonium sulfite is not a "fixed salt" such as is sodium sulfite since it may be removed from fermented media relatively easily. The use of this salt in fermentations for glycerol production should be feasible. It is probable that pH control of the fermenting medium by means of sulfur dioxide would be advisable.

6. It was shown that automatic alkaline pH-controlled fermentations could be run using aqueous solutions of sodium carbonate and sodium hydroxide as the controlling agents. The use of the sodium hydroxide solution for control was more satisfactory, however.

7. Calcium sulfite can be used in fermentations in order to increase the glycerol yield. An important advantage to the use of calcium sulfite for the production of glycerol by fermentation is the fact that it may be removed from the medium by filtration. Adjustment of pH before filtration is advisable. The solubility of calcium sulfite is a function of pH. Increasing the initial dextrose concentration in the fermentation medium decreases the percentage glycerol yield. The percentage yield of glycerol is an exponential function of the initial dextrose concentration. The fermentation time was found to be relatively short, from one to about four days, depending on the initial dextrose concentration. Filtration of a fermented medium containing calcium sulfite results in a filtered cake of solid calcium sulfite containing the culture of yeast. This cake may be used as a very active inoculum for subsequent fermentations. Contamination was very rare. The maximum glycerol yield approached 26 percent of the sugar weight as the initial sugar concentration approached zero.

8. Magnesium sulfite was found to act in a manner similar to calcium sulfite in fermentations for the production of glycerol. It was observed that the glycerol yield was greater in fermentations involving magnesium sulfite than in those employing calcium sulfite for the sugar concentrations studied. However, the maximum glycerol yield approached 26 percent of the sugar weight as the initial sugar concentration approached zero; this is the same limiting value as

that calculated for calcium sulfite.

9. It was shown that glycerol was formed in the presence of slightly soluble sulfites under acid conditions. The time for completion of the fermentations studied was relatively short.

10. The solubilities of calcium and magnesium sulfites are functions of pH. The bisulfite ion concentration is a function of the solubility of the sulfite and of pH. Toxicity is a function of bisulfite ion concentration; hence, it is a function of pH. Acetaldehyde fixation is a function of the bisulfite ion concentration; hence, it is also a function of pH. The glycerol yield is a function of the degree of acetaldehyde fixation. The variation of glycerol yield with pH was not outstanding, however. Acetaldehyde increases the solubility of calcium sulfite at a given pH. With a given concentration of acetaldehyde, the solubility of calcium sulfite decreases with increasing pH. The acetaldehyde-bisulfite complex is stable only under acid conditions and the stability decreases with increasing pH.

11. Two or more successive fermentations involving calcium or magnesium sulfite may be run on the same substrate provided solvents are removed between fermentations. Such a procedure increases the glycerol concentration in the medium thus making glycerol recovery methods generally more efficient.

12. It was shown that the acetaldehyde-bisulfite complex appreaches a maximum concentration in fermentation media utilizing calcium or magnesium sulfite for fixation of acetaldehyde. As the concentration of the complex increases, the concentration of acetaldehyde, or its equivalent, also increases, theoretically. The increased concentration of acetaldehyde results in a greater probability of its reduction to ethanol by means of glyceraldehyde phosphate; consequently a decreased yield of glycerol would be expected. The efficiency of acetaldehyde fixation should be greatest at the beginning of the fermentation before the acetaldehyde-bisulfite complex concentration is appreciable. A method of continuously removing the acetaldehyde from a sulfite fermentation medium should result in a greater glycerol yield. It may be possible to continuously remove the acetaldehyde from a circulated portion of the fermenting medium by a distillation, for example, under low pressure. An increase in pH would more easily allow distillation of the acetaldehyde from the medium. Following distillation, the acetaldehyde-free medium could be readjusted to the acid pH of the fermenting medium for further aldehyde fixation and glycerol formation by further fermentations and distillations.

LITERATURE CITED

Anderson, C. G. 1938. An introduction to bacteriological chemistry. The Williams and Wilkins Company, Baltimore.

Association of Official Agricultural Chemists. 1925. Official and tentative methods of analysis of the Association of Agricultural Chemists, 2nd edition. Association of Official Agricultural Chemists, Washington, D. C.

Barbet, E. A. 1928. A process for the utilization of alcoholic fermentation in order to form large quantities of glycerine by the use of sulfurous acid. British Patent 282,917. Jan. 5.

Benedict, S. R. 1931. The analysis of whole blood. II. The determination of sugar and of saccharoids (non-fermentable copper-reducing substances). J. Biol. Chem., 92:141-159.

Blish, M. J., and Sandstedt, R. M. 1933. An improved method for the estimation of flour diastatic value. Cereal Chem., 10:189-202.

Buchanan, R. E., and Fulmer, E. I. 1930a. The physiology and biochemistry of bacteria. Vol. II. The Williams and Wilkins Company, Baltimore.

Buchanan, R. E., and Fulmer, E. I. 1930b. The physiology and biochemistry of bacteria. Vol. III. The Williams and Wilkins Company, Baltimore.

Carothers, W. H., Hill, J. W., and Van Natta, F. J. L. 1933. Method of manufacturing fermentation glycerol. U. S. Patent 1,936,497. Nov. 21.

Cocking, A. T., and Lilly, C. H. 1922. Production of glycerin by fermentation. U. S. Patent 1,425,838. Aug. 15.

Connstein, W., and Lüdecke, K. 1919. Über Glycerin-Gewinnung durch Gärung. Ber., 52:1385-1391.

Connstein, W., and Ludecke, K. 1921. Process for manufacturing of propantriol from sugar. U. S. Patent 1,368,023. Feb. 8.

Connstein, W., and Lüdecke, K. 1924. Process for manufacturing of propantriol from sugar. U. S. Patent 1,511,754. Oct. 14.

Doak, B. W. 1939. A new method for clarification of plant extracts for the determination of reducing sugars. New Zealand J. Sci. Tech., 21B:90-95. Original not seen. Cited from C. A. 34: 2734 (1940).

Eoff, John R. Jr. 1918. Process for manufacturing glycerol. U. S. Patent 1,288,398. Dec. 17.

Eoff, John R. Jr., Linder, W. V., and Beyer, G. F. 1919. Production of glycerin from sugar by fermentation. J. Ind. Eng. Chem., 11:842-845.

Fulmer, E. I. 1930. The chemical approach to problems of fermentation. J. Ind. Eng. Chem., Ind. Ed., 22:1148-1150.

Fulmer, E. I., Hickey, R. J., and Underkofler, L. A. 1940. Determination of glycerol by oxidation with ceric sulfate in fermentation media containing dextrose. Ind. Eng. Chem., Anal. Ed., 12:729-730.

Fulmer, E. I., and Werkman, C. H. 1930. An index to the chemical action of microorganisms on the non-nitrogenous organic compounds. C. C. Thomas, Springfield.

Gehle, H. 1922. Vergärung von Zucker bei Gegenwart von Dinatriumsulfit nach Neuberg und Reinfurth. Biochem. Z. 132:566-588.

Giordani, M. 1932. Ricerche sulla fermentazione glicerica. Giorn. chim. ind. applicata, 14:597-600.

Golovin, P. V. 1927. Glycerol from molasses. Zhur. Sakharnoĭ Prom. 1:22-25. Original not seen. Cited from C. A. 23: 4527 (1929).

Gore, H. C., and Steele, H. K. 1935. Estimation of the saccharifying power of malt diastase. Adaption of the Hagedorn and Jensen method. Ind. Eng. Chem., Anal. Ed. 7:324-326.

Guymon, J. F. 1939. Relation of the structure of sugars to the chemism of the butyl-acetonic fermentation. Unpublished Ph. D. thesis, Iowa State College.

Haehn, H. 1938. Production of glycerin by fermentation. British Patent 488,464. July 7. C. A. 33:310 (1939).

Haehn, H. 1940. Production of glycerin by fermentation. U. S. Patent 2,189,793. Feb. 13.

Hagedorn, H. C., and Jensen, B. N. 1923. Zur Mikrobestimmung des Blutzuckers mittels Ferricyanid. Biochem. Z. 135:46-58.

Hesse, F. M. 1935. Production of glycerol from sugar. Rev. cubana azucar y alcohol 1:282-283. Original not seen. Cited from C. A. 30:2789 (1936).

Imperial Chemical Industries, Ltd., and Lilly, C. H. 1930. Glycerol production by fermentation. British Patent 349,192. March 18. C. A. 26:2010 (1932).

Iwata, Y. 1936. Fermentation process for obtaining glycerol from cane juice. Rept. Govt. Sugar Expt. Sta., Tainan, Formosa No. 3:76-92. Original not seen. Cited from C. A. 31:801 (1937).

Jaulmes, P., and Espezel, P. 1935. Le dosage de l' acétaldéhyde dans les vins et les spiritueux. Ann. fals. 28:325-335.

Kobel, M., and Tychowski, A. 1928. Biochemische Spaltung des Zuckers nach der zweiten Vergärungsform unter dem Einfluss von Carbaminsaurehydrazid und Thio-carbaminsaurehydrazid. Isolierung von Acetaldehyd und Glycerin. Biochem. Z. 199: 218-229.

Kobel, M. 1931. Die Bildung äquimolekularer Mengen von Glycerin und Brenztraubensäure bei der zellfreien Vergärung von Glucose. Biochem. Z. 243:406-415.

Kolthoff, I. M., Menzel, I. H., and Furman, N. H. 1929. Volumetric Analysis. Vol. II, John Wiley and Sons, Inc., New York.

Kolthoff, I. M., and Sandell, E. B. 1937. Textbook of quantitative inorganic analysis. The MacMillan Company, New York.

Krug, Wm. F., Jr., and McDermott, F. A. (to E. I. duPont de Nemours and Co.) 1935. Production of glycerol by fermentation. U. S. Patent 1,990,908. Feb. 12.

Kurbatova, U. S., and Shakin, A. N. 1936. Sulfite fermentation under conditions of repeated utilization of yeast. Biokhimiya 1:457-466. Original not seen. Cited from C. A. 31:7592 (1937).

Lawrie, J. W. (to E. I. duPont de Nemours and Co.) 1928. Glycerol by fermentation. U. S. Patent 1,678,150. July 24.

Lawrie, James W. 1928. Glycerol and the glycols. Amer. Chem. Soc. Monograph No. 44.

Lewis, G. N., and Randall, M. 1923. Thermodynamics. McGraw-Hill Book Company, Inc. New York.

Lilly, C. H. (to Imperial Chemical Industries, Ltd.) 1935. Glycerol production by fermentation. U. S. Patent 1,987,260. Jan. 8.

Lüdecke, K., and Lüdecke, N. 1929. Process for the production of glycerin. U. S. Patent 1,698,800. Jan. 15.

Lüdecke, K. 1938. Glycerol by fermentation. German Patent 658,047. April 27. C. A. 32:5992 (1938).

McDermott, Frank A. 1925. Glycerol by sugar fermentation in an alkaline medium. U. S. Patent 1,551,997. Sept. 1. C. A. 19:3347 (1925).

McDermott, F. A., (to E. I. du Pont de Nemours and Co.) 1929. Production of glycerol by fermentation. U. S. Patent 1,725,363. Aug. 20.

Mellor, J. W. 1930. A comprehensive treatise on inorganic and theoretical chemistry. Vol. X, pp. 282-285. Longmans, Green and Company, New York.

Munson, L. S., and Walker, P. H. 1906. The unification of reducing sugar methods. J. Am. Chem. Soc. 28:663-686.

Muzzey, A. K. 1932. Some aspects of the preparation of glycerine by fermentation with a laboratory study of fermentations using commercial yeasts and selected yeasts. Unpublished M. S. thesis, University of Pennsylvania.

Neuberg, Carl. 1913. Weitere Untersuchungen über die biochemische Umwandlung von Methylglyoxal in Milchsäure nebst Bemerkungen über die Entstehung der verschiedenen Milchsäuren in der Natur. Biochem. Z. 51:484-508.

Neuberg, C., and Hirsch, J. 1919. Über den Verlauf der alkoholischen Gärung bei alkalischer Reaktion. II. Gärung mit lebender Hefe in alkalischen Lösungen. Biochem. Z. 96:175-202.

Neuberg, C., and Hirsch, J. 1919. Die dritte Vergärungsform des Zuckers. Biochem. Z. 100:304-322.

Neuberg, C., and Hirsch, J., and Reinfurth, E. 1920. Die drei Vergärungsformen des Zuckers, ihre Zusammenhänge und Bilanz. Biochem. Z. 105:307-336.

Neuberg, C., and Kobel, M. 1930. Die Zerlegung von nicht phosphoryliertem Zucker durch Hefe unter Bildung von Glycerin und Brenztraubensäure. Biochem. Z. 229:446-454.

Neuberg, C., and Oertel, W. 1913. Studien über Methylglyoxalbildung. Biochem. Z. 55:495-503.

Neuberg, C., and Reinfurth, E. 1918. Natürliche und erzwungene Glycerin-bildung bei der alkoholischen Gärung. Biochem. Z. 92:234-266.

Neuberg, C., and Reinfurth, E. 1919. Weitere Untersuchungen über die korrelative Bildung von Acetaldehyd und glycerin bei der Zuckerspaltung and neue Beiträge zur Theorie der alkoholischen Gärung. Ber. 52:1677-1702.

Neuberg, C., and Schwenk, E. 1915. Veränderungen im Alkohol-und Aldehydgehalt von Hefen bei der Aufbewahrung und bei der Autolyse. Biochem. Z. 71:126-132.

Norddeutsche Hefeindustrie A. -G. 1938. Glycerol by fermentation. German Patent 655,177. Jan. 11. C. A. 32:2683 (1938).

Norddeutsche Hefeindustrie A. -G. 1938. Glycerol. German Patent 664,575. Aug. 29. C. A. 33:310 (1939).

Oppenheimer, C., and Stern, K. G. 1939. Biological oxidation. Nordemann Publishing Company, Inc., New York.

Owen, Wm. L. 1937. Blackstrap molasses as a raw material for biochemical industries. Facts about Sugar 32:142-144.

Owen, Wm. L. 1939. Blackstrap molasses as a raw material for biochemical industries. Russell Palmer, Publisher, New York.

Pasteur, L. 1858. Production constante de glycérine dans la fermentation alcoolique. Compt. rend. acad. sci. 46:857.

Pasteur, L. 1858. Nouvelles recherches sur la fermentation alcoolique. Compt. rend. acad. sci. 47:224.

Penkowski, J. 1929. Glycerin durch alkoholische Gärung. Chem.-techn. Übersicht 53:7.

Prescott, S. C., and Dunn, C. C. 1940. Industrial microbiology. McGraw-Hill Book Company, Inc., New York.

Rao, Y. K. R. 1937. Glycerol by fermentation of waste cane molasses. (Mandya). Proc. Soc. Biol. Chemists, India 2:38. Original not seen. Cited from C. A. 32:1856 (1938).

Shultz, A. S., Atkin, L. and Frey, C. N. 1940. The effect of bios on the nitrogen metobolism of yeast. I. Ammonia and carbamide. J. Biol. Chem. 135:267-271.

Shaffer, P. A., and Hartmann, A. F. 1921. The iodometric determination of copper and its use in sugar analysis. II. Methods for the determination of reducing sugars in blood, urine, milk, and other solutions. J. Biol. Chem. 45:365-390.

Shaffer, P. A., and Somogyi, M. 1933. Copper-iodometric reagents for sugar determination. J. Biol. Chem. 100:695-713.

Somogyi, M. 1937. Reagent for the copper-iodometric determination of sugar. J. Biol. Chem. 117:771-776.

Stephenson, Marjorie. 1939. Bacterial metabolism, 2nd edition. Longmans, Green and Co. New York.

Stiles, H. R., Peterson, W. H., and Fred, E. B. 1926. A rapid method for the determination of sugar in bacterial cultures. J. Bact. 12:427-439.

Takahashi, T., and Asai, T. 1933. Fermentation products of mucor. III. Production of glycerol and the effect of addition of sodium bisulfite and sodium carbonate. J. Agr. Chem. Soc. Japan. 9:443-448.

Tomoda, Y. 1928. Production of glycerol by fermentation. VI. Influence of sugar concentration on the yield of glycerol. Chem. News 137:265-266.

Tomoda, Y. 1929. Production of glycerol by fermentation. VII. The velocity of fermentation in the presence of sulfite. (Translated title.) J. Soc. Chem. Ind. (Japan) 32:229-303.

Tomoda, Y. 1929. A simple method for the determination of acetaldehyde. J. Soc. Chem. Ind. 48:76-77T.

Vokorny. 1913. Einwirkung des freien Ammoniaks auf die Hefe. Vergleich mit anderen Basen. Z. Spiritusind. 36:117.

Werkman, C. H. 1939. Bacterial dissimilation of carbohydrates. Bact. Rev. 3:187-227.

Williams, E. C. 1938. Modern petroleum research. Ind. Eng. Chem., News Ed. 16:630-633.

Williams, E. C., and Associates. 1940. Synthetic glycerine from petroleum. Chem. and Met. 47:834-838.

Williams, E. C., and Associates. 1941. Economic aspects of synthetic glycerine production. Chem. and Met. 48:87-89.

Wurtz, A. von, 1857. Ueber die künstliche bildung des Glycerine. Ann. 102:339-341.